Description

Methylotrophic Yeast Producing Mammalian Type Sugar Chain

Technical Field

The present invention provides a process for mass production of non-antigenic mammalian type glycoproteins comprising a sugar chain structure at their asparagine residues using a methylotrophic yeast wherein the sugar chain structure is identical to that produced by mammalian cells. More specifically, the present invention relates to a novel mutant yeast capable of producing a glycoprotein comprising a mammalian type sugar chain, which is created by introducing an α-1,2-mannosidase gene into a methylotrophic yeast having a mutation of sugar chain biosynthesizing enzyme genes, so that the α-1,2-mannosidase gene is highly expressed under the control of a potent promoter in the yeast, and the α-1,2-mannosidase exists in the endoplasmic reticulum (ER); and to a process for producing a glycoprotein comprising a mammalian type sugar chain wherein the process comprises culturing the methylotrophic yeast cells with a heterologous gene transferred thereinto in a medium and obtaining the glycoprotein comprising mammalian type sugar chains from the culture.

Background of the Invention

Yeast has been intensively studied as a host for production of foreign genes since establishment of yeast transformation systems. The use of a yeast for production of foreign proteins involves advantages in that molecular-genetic manipulation and culture of yeasts are as easy as those of prokaryotic organisms, and that yeasts bear eukaryotic type functions to allow post-translational modifications of proteins such as glycosylation. However, since production of proteins using *Saccharomyces cerevisiae* is low with exception of some successes, protein production systems using yeasts other than *Saccharomyces cerevisiae* have been developed, including systems using, for example, *Shizosaccharomyces pombe*, *Kluyveromyces lactis*, methylotrophic yeasts, or the like.

A methylotrophic yeast (or methanol-utilizing yeast), which can grow on methanol as a single carbon source, has been developed as a host for production of foreign proteins (K. Wolf (ed.) "Non Conventional Yeasts in Biotechnology" (1996)). This is because methods of culturing yeasts have been established in industrial scale and because the yeast has a potent promoter controlled by methanol. At that time when a methylotrophic yeast was discovered, the use thereof as SCP (Single Cell Protein) was studied and, as a result, a high-density culture technique at a dry cell weight of 100 g/L or more was established in an inexpensive culture medium, which contains minerals, trace elements, biotin, and carbon sources.

Researches on elucidation of a C1 compound-metabolic pathway, as well as on application of C1 compounds, revealed that a group of enzymes required for the methanol metabolism was strictly regulated by carbon sources. The methanol metabolism in a methanol-utilizing yeast generates formaldehyde and hydrogen peroxide from methanol and oxygen by alcohol oxidase in the first reaction. The generated hydrogen peroxide is decomposed into water and oxygen by catalase, while formaldehyde is oxidized to carbon dioxide by actions of formaldehyde dehydrogenase, S-formylglutathione hydrolase, and alcohol oxidase, and NADH generated during the oxidation is utilized as an energy source of the cell. At the same time, formaldehyde is condensed with xylulose-5-phosphate by dihydroxyacetone synthase, then converted into glyceraldehyde-3-phosphate and dihydroxyacetone, which subsequently enter the pentose phosphate pathway and serve as cell components.

Alcohol oxidase, dihydroxyacetone synthase, and formate dehydrogenase are not detected in the cell when it is cultured in the presence of glucose, but they are induced in the cell cultured in methanol, so that the amount of them is dozens of percentage of the total inner cell protein. Since the production of these enzymes is controlled at a transcription level, inducible expression of a foreign gene of interest is enabled under the regulation of promoters of the genes which encode the enzymes. The foreign gene expression system using a promoter for a methanol metabolizing enzyme gene has been estimated so highly among yeast expression systems due to its efficient production, with an example in which the expression

amount of a foreign gene was dozens of percentage of the total protein in cell or several g/L culture medium in secretion.

To date, four types of the transformation and foreign gene expression systems have been established in the methylotrophic yeasts: *Candida boidinii*, *Hansenula polymorpha*, *Pichia pastoris* and *Pichia methanolica*. Differences are recognized among the expression systems in terms of codon usage, expression regulation, and integration of expression plasmid, which provide characteristics of each expression system.

In the meantime, it is known that naturally occurring proteins are classified into two types, i.e., the one being a simple protein comprising amino acids alone, the other being a complex protein comprising sugar chains, lipids, phosphates or the like attached thereto, and that most of cytokines are glycoproteins. Recently, besides conventional analyses with lectin, new analyses using HPLC, NMR or FAB-MAS have been developed in analyzing sugar chain structures, by which new sugar chain structures of a glycoprotein have been found successively. On the other hand, studies on functional analysis of sugar chains lead to elucidation of the fact that the sugar chain plays an important role in lots of bio-mechanisms, such as intercellular recognition, molecular recognition, keeping of protein structures, contribution to protein activity, *in vivo* clearance, secretion, localization, etc.

For example, it has been revealed that erythropoietin (EPO), tissue plasminogen activator (TPA) or the like did not exhibit its inherent bioactivity when the sugar chains are removed (Akira Kobata, Tanpakushitsu-Kakusan-Koso, 36, 775-788 (1991)). Importance of sugar chains has been pointed out in erythropoietin, which was the first glycoprotein medicament in history produced by transgenic animal cells as the host. Specifically, the sugar chains of erythropoietin act in inhibitory manner against binding to receptor, whereas they have a decisive contribution to keeping of active structures and to improvement in *in vivo* pharmacokinetics, and are totally essential for expression of the pharmacological activity (Takeuchi and Kobata, Glycobiology, 1, 337-346 (1991)). Furthermore, high correlation between the structure, type and number of branches (i.e., the number of branches formed by GlcNAc attached to Man3GlcNAc2) of sugar chains and the pharmacological effect of erythropoietin has been found (Takeuchi et al., Proc. Natl. Acad. Sci. USA, 86, 7819-7822

(1989)). It was reported that a main cause of this phenomenon was that erythropoietin with immature branch structure is prone to occur its rapid clearance from the kidney, resulting in a shorter retention time in the body (Misaizu et al., Blood, 86, 4097-4104 (1995)). Another similar example is observed in serum glycoproteins including fetuin. That is, it was found that when removal of sialic acid at the end of a sugar chain leads to exposure of galactose, the galactose is recognized by lectin on the surface of liver cells, whereby the serum glycoprotein disappears promptly from the blood (Ashwell and Harford, Annu.Rev.Biochem., 51, 531-554 (1982); Morell et al., J.Biol.Chem., 243, 155-159 (1968)).

Glycoprotein sugar chains are largely classified into Asn-linked (N-linked), mucin type, O-GlcNAc type, GPI-anchored type, and proteoglycan type (Makoto Takeuchi, Glycobiology Series 5, Glycotechnology; edited by Akira Kibata and Senichiro Hakomori, Katsutaka Nagai, Kodansha Scientific, 191-208 (1994)), each of which has an intrinsic biosynthesis pathway and serves for individual physiological functions. Of them, for the biosynthesis pathway of Asn-linked sugar chains, there are many findings and detailed analyses.

Biosynthesis of Asn-linked sugar chains starts with synthesis of a precursor comprising N-acetylglucosamine, mannose and glucose on a lipid carrier intermediate, which precursor is converted to a specific sequence (Asn-X-Ser or -Thr) of a glycoprotein in the endoplasmic reticulum (ER). It is then subjected to processing (i.e., cleavage of glucose and specific mannose residues) to synthesize an M8 high-mannose type sugar chain comprising eight mannose residues and two N-acetylglucosamine residues (Man8GlcNAc2). The protein including high mannose type sugar chains is transported to the Golgi apparatus which undergoes a variety of modifications significantly different between yeast and mammal (Gemmill, T.R., Trimble, R.B., Biochim.Biophys.Acta., 1426, 227 (1999)).

In mammalian cells, in many cases, α-mannosidase I (α-1,2-mannosidase), an exomannosidase which cleaves an α-1,2-mannoside linkage, acts on high mannose type sugar chains to cut off several mannose residues. The sugar chain (Man5-8GlcNAc2) generated in this process is a sugar chain referred to as a high mannose type. N-acetylglucosaminyl transferase (GnT) I acts on an M5 high mannose type sugar chain (Man5GlcNAc2) from which three mannose residues have been cut off, to transfer one N-acetylglucosamine residue

to the sugar chain, resulting in formation of a sugar chain comprising GlcNAcMan5GlcNAc2. The thus formed sugar chain is referred to as a hybrid type. Further, when α-mannosidase II and GnT II act, the sugar chain structure GlcNAc2Man3GlcNAc2, referred to as a complex type, is formed. Thereafter, a variety of mammalian type sugar chains are formed through the action of a group of ten-odd glycosyltransferase enzymes, by which addition of N-acetylglucosamine, galactose, sialic acid, etc. occurs (Fig. 1).

Accordingly, the mammalian type sugar chain as defined in this application means an N-linked (or Asn-linked) sugar chain present in mammals, which is generated in the sugar chain biosynthesis process of mammals. Specifically, they include an M8 high mannose type sugar chain represented by Man8GlcNAc2; an M5, M6 or M7 high mannose type sugar chain represented by Man5GlcNAc2, Man6GlcNAc2 or Man7GlcNAc2, respectively, generated from Man8GlcNAc by action of α-mannosidase I; a hybrid type sugar chain represented by GlcNAcMan5GlcNAc2 generated from Man5GlcNAc2 by action of GlcNAc transferase-I (GnT-I); a double-stranded complex type sugar chain represented by GlcNAc2Man3GlcNAc2 generated from GlcNAcMan5GlcNAc2 by action of α-mannosidase-I and GlcNAc transferase-II (GnT-II); and a double-stranded complex type sugar chain represented by Gal2GlcNAc2Man3GlcNAc2 generated from GlcNAc2Man3GlcNAc2 by action of galactosyl transferase (GalT).

In mammals, any of high mannose type, hybrid type and complex type sugar chains can be found. In one case, sugar chains to be attached are different depending on a protein, or in another, different types of sugar chains are attached within a protein. These sugar chains exhibit important functions, such as biosynthesis of glycoproteins, sorting within a cell, concealment of antigenicity, *in vivo* stability, organ-targeting properties, and the like, depending on the type or class of sugar chains attached to a glycoprotein (Tamao Endo, Tosa Kogaku (Sugar chain engineering), Sangyo Chosakai, 64-72 (1992)).

On the other hand, in yeast a mannan-type sugar chain (outer sugar chain) is produced, in which several to 100 or more mannose residues are attached to M8 high mannose type sugar chain. For example, the biosynthesis of outer sugar chains in *Saccharomyces cerevisiae* known as baker's yeast or laboratory yeast is considered to proceed along a pathway as shown

in Fig. 2 (Ballou et al., Proc. Natl. Acad. Sci. USA, 87, 3368-3372 (1990)). That is, a reaction for initiating elongation begins in which a mannose is first attached to M8 high mannose type sugar chain through α-1,6 linkage (Fig. 2, Reaction I, B). The enzyme performing this reaction is clarified as a protein encoded by *OCH1* gene (Nakayama et al., EMBO J., 11, 2511-2519 (1992)). Further, sequential elongation of mannose by α-1,6-linkage reaction (Fig. 2, II), forms a poly α-1,6-mannose linkage being the backbone of an outer sugar chain (Fig. 2, E). The α-1,6-mannose linkage sometimes contains a branch of α-1,2-linked mannose (Fig. 2: C, F, H), and additionally, α-1,3-linked mannose is attached to the end of the branched α-1,2-linked mannose chain (Fig. 2: D, G, H, I). The addition of the α-1,3-linked mannose is caused by a *MNN1* gene product (Nakanishi-Shindo et al., J. Biol. Chem., 268, 26338-26345 (1993)). Formation of an acidic sugar chain, in which mannose-1-phosphate has been attached to high mannose type sugar chain moieties and outer chain moieties, is known as well (Fig. 2, *; a possible phosphorylation site corresponding to * in the above formula (I)). This reaction was found to be caused by a protein encoded by *MNN6* gene (Wang et al., J. Biol. Chem., 272, 18117-18124 (1997)). Further, a gene (*MNN4*) coding for a protein positively regulating the transfer reaction was clarified (Odani et al., Glycobiology, 6, 805-810 (1996); Odani et al., FEBS Letters, 420, 186-190 (1997)).

Production of substances using microorganisms including yeast has some advantages as mentioned above, such as low production costs and utilizing culture technology developed as fermentation engineering, as compared with the production of substances using animal cells. There is a problem, however, that microorganisms cannot attach sugar chains with the same structure as human glycoprotein. Specifically, glycoproteins from cells of an animal including human have a variety of mucin type sugar chains in addition to three kinds of Asn-linked sugar chains, i.e., complex type, hybrid type and high mannose type as shown in Fig. 1, while the Asn-linked sugar chain whose attachment is observed even in baker's yeast (*Saccharomyces cerevisiae*), is only a high mannose type, and a mucin type is attached only to a sugar chain mainly composed of mannose.

Such sugar chains of yeast may produce a heterogeneous protein product resulting in difficulties in purification of the protein or in reduction of specific activity (Bekkers et al.,

Biochim. Biophys. Acta, 1089, 345-351 (1991)). Furthermore, since the structure of the sugar chains significantly differ, glycoproteins produced by yeast may not have the same detectable biological activity as those of the mammalian origin, or have strong immunogenicity to a mammal, etc. Thus, yeast is unsuitable as a host for producing useful glycoproteins from mammalian origin, and in general microorganisms are not suitable for DNA recombinant production of a glycoprotein, such as erythropoietin as described above, in which sugar chain has an important function. Indeed, for production of erythropoietin, Chinese hamster ovary (CHO) cells are used.

Thus, it is expected that the sugar chain of a glycoprotein not only has a complicated structure but also plays an important role in expression of biological activity. However, since the correlation of the structure of sugar chain with biological activity is not necessarily clear, development of the technology, which enables to freely modify or control the structure (the type of sugar, a linking position, chain length, etc.) of a sugar chain attached to a protein moiety, is needed. When developing a glycoprotein especially as medicament, the structure and function analyses of the glycoprotein become important. Under these circumstances, the development of yeast, which can produce a glycoprotein with biological activity equivalent to that of the mammalian origin, i.e., a glycoprotein comprising a mammalian type sugar chain, is desired by the academic society and the industrial world.

In order to produce a mammalian type sugar chain using yeast, it is important to prepare a mutant having the sugar chain biosynthesis system, which does not comprise a reaction as mentioned above of attaching a lot of mannose residues to modify the glycoprotein sugar chain as seen particularly in yeast; in which no outer sugar chains are attached; and the synthesis of sugar chains generates M5 high mannose type sugar chain. Subsequently, M8 high mannose type sugar chain, a precursor for this mammalian type sugar chain, might be produced by introducing biosynthetic genes for the mammalian type sugar chain into the mutant yeast.

To obtain a glycoprotein lacking outer sugar chains, use of a mutant strain deficient in enzymes for producing outer sugar chains in yeast, particularly a mutant of *Saccharomyces cerevisiae*, has been studied so far. Methods to obtain such a deficient mutant strain include

obtaining a gene mutant by chemicals, ultraviolet irradiation or natural mutation, or obtaining it by artificial disruption of a target gene.

As to the former methods, there are many reports thereon. For example, *mnn2* mutant is defective in the step of branching which causes α-1,2 linkage from the α-1,6 backbone of an outer sugar chain, and *mnn1* mutant is defective in the step of producing α-1,3-linked mannose at the end of the branch. However, these mutants do not have defects in α-1,6 mannose linkage as the backbone of outer sugar chains and so they produce a long outer sugar chain in length. Mutants like *mnn7, 8, 9, 10* mutants have been isolated as mutants having only about 4 to 15 molecules of the α-1,6 mannose linkage. In these mutants, the outer sugar chains are merely shortened, but the elongation of high mannose type sugar chains does not stop (Ballou et al., J. Biol. Chem., 255, 5986-5991 (1980); Ballou et al., J. Biol. Chem., 264, 11857-11864 (1989)). Defects in the addition of outer sugar chains are also observed in, for example, secretion mutants such as *sec18* in which the transportation of a protein from endoplasmic reticulum to Golgi apparatus is temperature-sensitive. However, in a *sec* mutant, since the secretion of a protein itself is inhibited at a high temperature, the *sec* mutant is not suitable for secretion and production of glycoproteins.

Accordingly, since these mutants cannot completely biosynthesize the high mannose type sugar chain of interest, they are considered unsuitable as host yeast for producing a mammalian type sugar chain.

On the other hand, as to the latter, the deficient mutant strain in which a plurality of target genes have been disrupted can be established by development of genetic engineering techniques in recent years. Specifically, through *in vitro* operation, a target gene DNA on plasmid is first fragmentated or partially deleted, and an adequate selectable marker DNA is inserted at the fragmented or deleted site to prepare a construct in which the selectable marker is sandwiched between upstream and downstream regions of the target gene. Subsequently, the linear DNA having this structure is transferred into a yeast cell to cause two homologous recombinations at portions homologous between both ends of the introduced fragment and the target gene on chromosome, thereby substituting the target gene with a DNA construct in

which the selectable marker has been sandwiched (Rothstein, Methods Enzymol., 101, 202-211 (1983)).

Molecular cloning of a yeast strain deficient in outer sugar chain has already been described by Jigami et al. in Japanese Patent Publication (Kokai) No. 6-277086A (1994) and No. 9-266792A (1997). Jigami et al. succeeded in cloning of the *S. cerevisiae OCH1* gene (which expresses α-1,6-mannosyl transferase), the *OCH1* enzyme being assumed to be a key enzyme for elongation of the α-1,6 linked mannose. The glycoprotein of the *OCH1* gene knockout mutant (*Δoch1*) had three types of attached sugar chains, i.e., Man8GlcNAc2, Man9GlcNAc2 and Man10GlcNAc2. Of them, the Man8GlcNAc2 chain had the same structure (i.e., the structure shown in Fig. 2A) as the ER core sugar chain which was common between *S. cerevisiae* and mammalian cell, while the Man9GlcNAc2 and Man10GlcNAc2 chains had a structure where α-1,3-linked mannose was attached to this ER core sugar chain [Nakanish-Shindo, Y., Nakayama, K., Tanaka, A., Toda, Y. and Jigami, Y., (1994), J.Biol.Chem.]. Furthermore, a *S. cerevisiae* host which can attach only the Man8GlcNAc2 chain having the same structure as the ER core sugar chain, which structure is common between *S. cerevisiae* and mammalian cell, was successfully produced by preparing a *Δoch1mnn1* dual mutant and inhibiting the α-1,3-linked mannose transfer at the end. It is supposed that this *Δoch1mnn1* double mutant serves as a host useful in case where the mammalian glycoprotein, which has a high mannose type sugar chain, is produced by DNA recombinant technology [Yoshifumi Jigami (1994) Tanpakushitsu-Kakusan-Koso, 39, 657].

It was found, however, that sugar chains of the glycoprotein produced by the double mutant (*Δoch1mnn1*) described in Japanese Patent Publication (Kokai) No. 6-277086 (1994) comprised acidic sugar chains containing a phosphate residue. This acidic sugar chain has a structure which is not present in sugar chains of mammals including human, and it is likely to be recognized as a foreign substance in mammal, thereby exhibiting antigenicity (Ballou, Methods Enzymol., 185, 440-470 (1990)). Hence, a quadruple mutant (as described in Japanese Patent Publication (Kokai) No. 9-266792A (1997)) was constructed in which the functions of a gene for positively regulating the transfer of mannose-1-phosphate (*MNN4*) and of a mannose transferase gene for performing the elongation reaction for an O-linked sugar

chain (*KRE2*) have been disrupted. It was revealed that the sugar chain of a glycoprotein produced by the yeast strain described therein had the M8 high mannose type sugar chain of interest. It was further found that a strain in which *Aspergillus saitoi*-derived α-1,2-mannosidase gene is transferred to a yeast cell where a gene involved in the particular sugar chain biosynthesis system of yeast has been disrupted, had a high mannose type sugar chain (Man5-8GlcNAc2) in which one to several mannose residues were cleaved (Chiba et al., J. Biol. Chem., 273, 26298-26304 (1998)). Furthermore, they attempted production of a mammalian type glycoprotein in yeast by transfer of a gene involved in the mammalian sugar chain biosynthesis system into this prepared strain (PCT/JP 00/05474). However, despite that an α-1,2-mannosidase gene was expressed using a promoter for glyceraldehyde-3-phosphate dehydrogenase gene which is considered to be the highest in the expression amount as a constitutive expression promoter according to the disclosure, the conversion efficiency to Man5GlcNAc2 by carboxypeptidaseY (CPY) in the cell wall-derived mannoprotein is as low as 10-30% and so it is hard to say that its application to various glycoproteins is sufficiently prospective, although the rate of conversion to a high mannose type sugar chain (Man5GlcNAc2) was almost 100% in FGF as a foreign protein.

Separately, Schwientek et al. reported on the expression of the activity of human β-1,4-galactosyl transferase gene in *S. cerevisiae* in 1994 [Schwientek, T. and Ernst, J.F., Gene, 145, 299 (1994)]. Similarly, Krezdrn et al. achieved the expression of the activity of human β-1,4-galactosyl transferase gene and α-2,6-sialyl transferase in *S. cerevisiae* [Krezdrn, C.H.et al., Eur.J.Biochem.220, 809 (1994)].

However, when these findings are tried to be applied to other yeast, various problems arise. First of all, it is known that yeasts themselves have various sugar chain structures (K. Wolf et al., Nonconventional Yeasts in Biotechnology (1995)).

For example, a divided yeast *Schizosaccharomyces pombe* contains galactose. *Kluyveromyces lactis* has GlcNAc. Both the methylotrophic yeast *Pichia pastoris* and the pathogenic yeast *Candida albicans* have been confirmed to contain β-mannoside linkage. Even yeasts having xylose and rhamnose as sugar chain components exist (Biochim.et Biophy.Acta, 1426, 1999, 227-237).

In fact, no yeasts capable of producing mammalian type sugar chains have been obtained except *Saccharomyces cerevisiae* as reported by Jigami et al. Also, although use of a methylotrophic yeast as the host for producing a foreign protein was exemplified in Japanese Patent Publication (Kokai) No. 9-3097A (1997), substantially no other example has been given.

In Japanese Patent Publication (Kokai) No. 9-3097A (1997), a homologue of *Pichia pastoris OCH1* gene and a *Pichia pastoris* mutant strain in which the *OCH1* gene was knockout were prepared, to obtain from them a modified methylotrophic yeast strain whose ability to extend a sugar chain was inhibited as compared with natural methylotrophic yeast strain. This publication, however, provides only information on SDS-PAGE of the produced glycoprotein, and no such support as structural analysis data. That is, it did not actually identify the activity but only pointed out about possibility of being α-1,6-mannosyl transferase. In fact, although *HOC1* gene (GenBank accession number; U62942), which is an *OCH1* gene homologue, exists also in *Saccharomyces cerevisiae*, the activity and function thereof are unknown at present.

Moreover, in the same publication a sugar chain having β-mannoside linkage in *P. pastoris* was identified, but it did not describe about the structure of the chain in any way. Indeed, structural analysis of the sugar chain was neither performed nor identified the produced sugar chain. So, it was not demonstrated whether or not the obtained gene is actually the *OCH1* gene, and whether or not the sugar chain of the knockout strain was a mammalian type. Accordingly, one cannot safely say that the technique disclosed in Japanese Patent Publication (Kokai) No. 9-3097A (1997) produces a mammalian type sugar chain bearing glycoprotein and is sufficient as the production system that can be adapted for production of medicaments.

There is also a study using a filamentous fungus *Trichoderma reesei* by Maras et al. as an attempt to produce a mammalian type sugar chain using a microorganism other than yeast (USP 5,834,251). The disclosed method comprises making α-1,2-mannosidase and GnT-I to act on filamentous fungus and yeast to synthesize a hybrid type sugar chain (i.e., GN1Man5 sugar chain).

Filamentous fungi inherently express α-1,2-mannosidase, and consequently it is believed that little sugar chain modification occurs as compared with the case of yeast. On the other hand, since yeast attaches a particular outer sugar chain, all sugar chains are not obtained as Man5 by the procedure in which only α-1,2-mannosidase is introduced. In fact, produced in *Saccharomyces cerevisiae* as disclosed in this patent publication was a mixture of Man5 as the final product with sugar chains of Man6 or more as partial decomposition products, which mixture is produce by action of the outer sugar chain synthesizing gene *OCH1*, as described by Jigami or Chiba et al. (su*pra*). It would accordingly be hard to say that the mammalian type sugar chain was produced in *S. cerevisiae*, and so this purpose cannot be attained without disrupting a sugar chain biosynthesizing gene of yeast. Maras et al. did not mention the gene disruption in the sugar chain biosynthesis system inherent to yeast at all, so obviously this technique could not be applied to yeasts (*Pichia pastoris*, *Hansenula polymorpha*, *Kluyveromyces lactis*, *Saccharomyces cerevisiae*, *Yarrowia lipolytica*). Moreover, Maras et al. refers to RNaseB as a heterologous expression protein in the Examples, but RNaseB has originally a high mannose type sugar chain of Man5 or Man6. Many of the sugar chains of the animal cell origin are complex type sugar chains having complicated structures, and many of glycoproteins such as cytokines expected to be applied to medicaments etc. have complex type sugar chains. In fact, it is known that the sugar chain structure changes greatly depending on kinds of foreign glycoproteins expressed (Method in Molecular Biologylogy, 103, 95-105 (1998)). Therefore, it is considered inappropriate to use as an example RNaseB which is a glycoprotein originally having a high mannose type sugar chain, in the application to glycoproteins having complex type sugar chains.

Furthermore, filamentous fungi are commonly used for the production of industrial enzymes, food enzymes, etc., and the transformation system is established, and production of enzymes by DNA recombinant technology has also been conducted. Nevertheless, there are the following disadvantages:

1) Since the protease activity is very strong, proteins produced are prone to receive limited proteolysis.

2) Since the fungi produce many proteins secreted outside the cell, they are unsuitable for the production of proteinous medicaments where homogeneity would be required.

Ogataea minuta as defined in the present invention is a strain once referred to as *Pichia minuta* or *Hansenulla minuta*, and was named *Ogataea minuta* by Ogata et al. (Biosci. Biotecnol. Biochem., 58, 1245-1257 (1994)). *Ogataea minuta* produces significant amounts of alcohol oxidase, dihydroxyacetone synthase and the formate dehydrogenase within the cell as in other methylotrophic yeasts, but nothing was known about the genes relating to these methanol utilization enzyme nor about sugar chain structures of this yeast.

Under the above-mentioned circumstances, the object of the present invention is to solve the above-described problems in production of glycoproteins in yeast, and to provide a process for mass production of non-antigenic mammalian type sugar chains and glycoproteins containing the sugar chains using a methylotrophic yeast wherein the sugar chain structures are identical to those of sugar chains as produced in human and other mammalian cells.

Disclosure of the Invention

For the purpose of constructing a production technique of glycoproteins having mammalian cell compatible sugar chain structures using a methylotrophic yeast, we conducted intensive researches to achieve the above-mentioned object. Consequently, we have found that sugar chains in *Ogataea minuta*, which is a kind of methylotrophic yeast, comprises mainly α-1,2-mannoside linkage, by NMR analysis of the cell wall sugar chain and by α-1,2-mannosidase digestion test, and further that glycoproteins having mammalian type sugar chains can be obtained by introducing an α-1,2-mannosidase gene into a mutant strain comprising mutated sugar chain biosynthesizing enzyme genes (for example, an *OCH1* gene (α-1,6-mannosyl transferase) knockout mutant, which is considered to be a key enzyme for the elongation reaction where mannose residues attach to an M8 high mannose type sugar chain one by one via α-1,6 linkage), and expressing it under the control of a potent promoter such as methanol-inducible promoter, followed by culturing the *Ogataea minuta* transformed with a heterologous gene in a culture medium, thereby to obtain a glycoprotein from the culture. By this finding was completed the present invention. Thus, it was found that a mammalian type

sugar chain could be produced without disrupting *MNN1* and *MNN4* genes in *Saccharomyces cerevisiae*.

In summary, the invention comprises:

1) A methylotrophic yeast strain producing a mammalian type sugar chain, obtained by introducing an α-1,2-mannosidase gene into a mutant strain comprising a mutated sugar chain biosynthesizing enzyme gene (for example, an *OCH1* gene (α-1,6-mannosyl transferase) knockout mutant, which is considered to be a key enzyme for the elongation reaction where mannose residues attach to an M8 high mannose type sugar chain one by one via α-1,6 linkage), and expressing it under the control of a potent promoter such as methanol-inducible promoter;

2) A process of producing a glycoprotein comprising a mammalian type sugar chain, comprising culturing in a culture medium the yeast strain bred by introducing heterologous genes into a mutant yeast which comprises mutated sugar chain biosynthesizing enzyme genes and expressing these genes, and obtaining the glycoprotein comprising a mammalian sugar chain from the culture; and

3) A glycoprotein comprising a mammalian type sugar chain, produced by this production process.

More specifically, the invention provides the following 1 to 122.

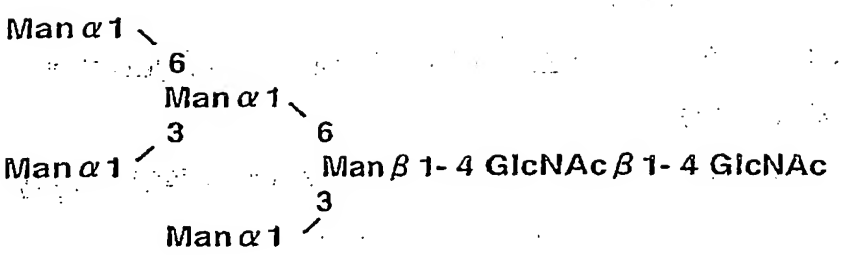
1. A process for producing a methylotrophic yeast capable of producing a mammalian type sugar chain, which comprises the steps of:

1) disrupting an *OCH1* gene which encodes α-1,6-mannosyl transferase, in a methylotrophic yeast; and

2) introducing an α-1,2-mannosidase gene into the yeast and expressing it therein.

2. A process according to (1), wherein the mammalian type sugar chain is represented by the following structural formula ($Man_5GlcNAc_2$):

Structural Formula 2



3. A process according to (1) or (2), wherein the methylotrophic yeast belongs to the genus *Pichia*, *Hansenula*, *Candida*, or *Ogataea*.

4. A process according to (1) or (2), wherein the methylotrophic yeast is *Ogataea minuta*.

5. A process according to any one of (1) to (4), wherein the methylotrophic yeast is a strain from *Ogataea minuta* strain IFO 10746.

6. A process according to any one of (1) to (5), wherein the α-1,2-mannosidase gene is expressed under the control of a methanol-inducible promoter.

7. A process according to (6), wherein the methanol-inducible promoter is a promoter of an alcohol oxidase (*AOX*) gene.

8. A process according to (7), wherein the alcohol oxidase (*AOX*) gene is from *Ogataea minuta*.

9. A process according to any one of (1) to (8), characterized in that the α-1,2-mannosidase gene to be introduced is attached to a yeast endoplasmic reticulum (ER) retention signal (HDEL).

10. A process according to any one of (1) to (9), wherein the α-1,2-mannosidase gene is from *Aspergillus saitoi*.

11. A process according to any one of (1) to (10), which further comprises a step of transforming a heterologous gene into the yeast.

12. A process according to (11), wherein the heterologous gene is transferred using an expression vector and is expressed in the yeast.

13. A process according to (12), wherein the expression vector comprises a methanol-inducible promoter.

14. A process according to (13), wherein the methanol-inducible promoter is a promoter of an alcohol oxidase (*AOX*) gene.

15. A process according to (14), wherein the alcohol oxidase (*AOX*) gene is from *Ogataea minuta*.

16. A process according to (12), wherein the expression vector comprises a promoter of a glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene.

17. A process according to any one of (11) to (16), wherein 20 % or more of N-linked sugar chains produced of the protein encoded by a heterologous gene is the mammalian type sugar chain represented by Structural Formula 2.

18. A process according to any one of (11) to (16), wherein 40 % or more of N-linked sugar chains produced of the protein encoded by a heterologous gene is the mammalian type sugar chain represented by Structural Formula 2.

19. A process according to any one of (11) to (16), wherein 60 % or more of N-linked sugar chains produced of the protein encoded by a heterologous gene is the mammalian type sugar chain represented by Structural Formula 2.

20. A process according to any one of (11) to (16), wherein 80 % or more of N-linked sugar chains produced of the protein encoded by a heterologous gene is the mammalian type sugar chain represented by Structural Formula 2.

21. A process according to any one of (11) to (20), wherein the protein encoded by a heterologous gene is from humans.

22. A process according to any one of (11) to (21), wherein the protein encoded by a heterologous gene is an antibody or a fragment thereof.

23. A methylotrophic yeast produced by a process according to any one of (1) to (22).

24. A process for producing a protein encoded by a heterologous gene, wherein the process comprises culturing the methylotrophic yeast of (23) in a medium to obtain the protein encoded by a heterologous gene comprising a mammalian type sugar chain from the culture.

25. A protein comprising a mammalian type sugar chain encoded by a heterologous gene, wherein the protein is produced by the process of (24).

26. An orotidine-5'-phosphate decarboxylase (*URA3*) gene DNA encoding an amino acid sequence substantially represented by SEQ ID NO:16.

27. A URA*3* gene DNA comprising a nucleotide sequence substantially represented by SEQ ID NO:15.

28. A recombinant expression vector substantially comprising the gene DNA of (26) or (27) or a fragment thereof as a selectable marker.

29. An *Ogataea minuta* strain transformed with a recombinant expression vector of (28).

30. An *Ogataea minuta* strain according to (29), the strain being from the strain IFO 10746.

31. A phosphoribosyl-amino-imidazole succinocarboxamide synthase (*ADE1*) gene DNA encoding an amino acid sequence substantially represented by SEQ ID NO:28.

32. An *ADE1* gene DNA comprising a nucleotide sequence substantially represented by SEQ ID NO:27.

33. A recombinant expression vector substantially comprising the gene DNA of (31) or (32) or a fragment thereof as a selectable marker.

34. An *Ogataea minuta* strain transformed with the recombinant expression vector of (33).

35. An *Ogataea minuta* strain according to (34), the strain being from the strain IFO 10746.

36. An imidazole-glycerol-phosphate dehydratase (*HIS3*) gene DNA encoding an amino acid sequence substantially represented by SEQ ID NO:100.

37. An *HIS3* gene DNA comprising a nucleotide sequence substantially represented by SEQ ID NO:99.

38. A recombinant expression vector substantially comprising the gene DNA of (36) or (37) or a fragment thereof as a selectable marker.

39. A *Ogataea minuta* strain transformed with a recombinant expression vector of (38).

40. An *Ogataea minuta* train according to (39), the strain being from the strain IFO 10746.

41. A 3-isopropylmalate dehydrogenase (*LEU2*) gene DNA encoding an amino acid sequence substantially represented by SEQ ID NO:108.

42. A *LEU2* gene DNA comprising a nucleotide sequence substantially represented by SEQ ID NO:107.

43. A recombinant expression vector substantially comprising the gene DNA of (41) or (42) or a fragment thereof as a selectable marker.

44. An *Ogataea minuta* strain transformed with the recombinant expression vector of (43).

45. An *Ogataea minuta* stain according to claim 44, the strain being from the IFO 10746.

46. An α-1,6-mannosyl transferase (*OCH1*) gene DNA encoding an amino acid sequence substantially represented by SEQ ID NO:43.

47. An *OCH1* gene DNA comprising a nucleotide sequence substantially represented by SEQ ID NO:42.

48. An *Ogataea minuta* strain wherein the gene of (46) or (47) has been disrupted.

49. An *Ogataea minuta* strain according to (48), the strain being from the strain IFO 10746 strain .

50. A proteinase A (*PEP4*) gene DNA encoding an amino acid sequence substantially represented by SEQ ID NO:52.

51. A *PEP4* gene DNA comprising a nucleotide sequence substantially represented by SEQ ID NO:51.

52. An *Ogataea minuta* strain wherein the gene of (50) or (51) has been disrupted.

53. An *Ogataea minuta* strain according to (52), the strain being from the strain IFO 10746.

54. A proteinase B (*PRB1*) gene DNA encoding an amino acid sequence substantially represented by SEQ ID NO:58.

55. A *PRB1* gene DNA comprising a nucleotide sequence substantially represented by SEQ ID NO:57.

56. An *Ogataea minuta* strain wherein the gene of (54) or (55) has been disrupted.

57. An *Ogataea minuta* strain according to (56), the strain being from the strain IFO 10746.

58. A *YPS1* gene DNA encoding an amino acid sequence substantially represented by SEQ ID NO:116.

59. A *YPS1* gene DNA comprising a nucleotide sequence substantially represented by SEQ ID NO:115.

60. An *Ogataea minuta* strain wherein the gene of (58) or (59) has been disrupted.

61. An *Ogataea minuta* strain according to (60), the strain being from the strain IFO 10746.

62. A process for producing a protein encoded by a heterologous gene, wherein the heterologous gene is transferred into the *Ogataea minuta* strain of (60) or (61).

63. A process according to (62), wherein the heterologous gene encodes an antibody or a fragment thereof.

64. A process for preventing from decomposition of an antibody or a fragment thereof, comprising disrupting a *YPS1* gene in a methylotrophic yeast.

65. A process according to (64), wherein the methylotrophic yeast is an *Ogataea minuta* strain.

66. A process according to (65), wherein the *Ogataea minuta* strain is from the strain IFO 10746.

67. A process according to any one of (64) to (66), wherein the class of the antibody is IgG.

68. A process according to (67), wherein the subclass of the antibody is IgG1.

69. A process according to any one of (64) to (68), wherein the antibody is a human antibody.

70. A *KTR1* gene DNA encoding an amino acid sequence substantially represented by SEQ ID NO:64.

71. A *KTR1* gene DNA comprising a nucleotide sequence substantially represented by SEQ ID NO:63.

72. An *Ogataea minuta* strain wherein the gene of (70) or (71) has been disrupted.

73. An *Ogataea minuta* strain according to (72), the strain being from the strain IFO 10746.

74. An *MNN9* gene DNA encoding an amino acid sequence substantially represented by SEQ ID NO:70.

75. An *MNN9* gene DNA comprising a nucleotide sequence substantially represented by SEQ ID NO:69.

76. An *Ogataea minuta* strain wherein the gene of (74) or (75) has been disrupted.

77. An *Ogataea minuta* strain according to claim 76, the strain being from the strain IFO 10746.

78. An alcohol oxidase (*AOX*) gene DNA encoding an amino acid sequence substantially represented by SEQ ID NO:78.

79. An *AOX* gene DNA comprising a nucleotide sequence substantially represented by SEQ ID NO:77.

80. A DNA comprising a promoter of alcohol oxidase (*AOX*), wherein the DNA comprises a nucleotide sequence substantially represented by SEQ ID NO:79.

81. A DNA comprising a terminator of alcohol oxidase (*AOX*), wherein the DNA comprises a nucleotide sequence substantially represented by SEQ ID NO:80.

82. A gene expression cassette comprising a DNA comprising a promoter as defined in (80), a heterologous gene, and a DNA comprising a terminator as defined in (81).

83. A recombinant expression vector comprising a gene expression cassette of (82).

84. An *Ogataea minuta* strain transformed with the recombinant expression vector of (83).

85. An *Ogataea minuta* strain according to (84), the strain being from the strain IFO 10746.

86. A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene DNA encoding an amino acid sequence substantially represented by SEQ ID NO:6.

87. A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene DNA comprising a nucleotide sequence substantially represented by SEQ ID NO:5.

88. A DNA comprising a promoter of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), wherein the DNA comprises an amino acid sequence substantially represented by SEQ ID NO:7.

89. A DNA comprising a terminator of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), wherein the DNA comprises an amino acid sequence substantially represented by SEQ ID NO:8.

90. A gene expression cassette comprising a DNA comprising a promoter as defined in (88), a heterologous gene, and a DNA comprising a terminator as defined in (89).

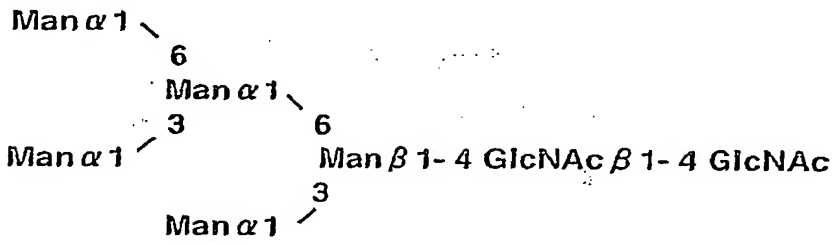
91. A recombinant expression vector comprising the gene expression cassette of (90).

92. An *Ogataea minuta* strain transformed with a recombinant expression vector of (91).

93. An *Ogataea minuta* strain according to claim 92, the strain being from the strain IFO 10746.

94. A process for producing an *Ogataea minuta* strain, which is capable of producing a mammalian type sugar chain represented by the following structural formula ($Man_5GlcNAc_2$):

Structural Formula 2



comprising a step of disrupting *OCH1* gene (SEQ ID NO:42) in the *Ogataea minuta* strain.

95. A process of (94), wherein the *Ogataea minuta* strain is from the strain IFO 10746.

96. A process according to (94) or (95), which further comprises a step of disrupting at least one gene selected from the group consisting of a URA*3* gene comprising the nucleotide sequence represented by SEQ ID NO:15, an *ADE1* gene comprising the nucleotide sequence represented by SEQ ID NO:27, an *HIS3* gene comprising the nucleotide sequence represented by SEQ ID NO:99, and a *LEU2* gene comprising the nucleotide sequence represented by SEQ ID NO:107.

97. A process according to any one of (94) to (96), which further comprises a step of disrupting at least one gene selected from the group consisting of a *PEP4* gene comprising the

nucleotide sequence represented by SEQ ID NO:51, a *PRB1* gene comprising the nucleotide sequence represented by SEQ ID NO:57, and a *YPS1* gene comprising the nucleotide sequence represented by SEQ ID NO:115.

98. A process according to any one of (94) to (97), which further comprises a step of disrupting a *KTR1* gene comprising the nucleotide sequence represented by SEQ ID NO:63 and/or an *MNN9* gene comprising the sequence represented by SEQ ID NO:69.

99. A process according to any one of (94) to (98), which further comprises a step of introducing and expressing an α-1,2-mannosidase gene from *Aspergillus saitoi*.

100. A process according to (99), wherein the α-1,2-mannosidase gene is transferred into the vector of (83) and expressed.

101. A process according to any one of (94) to (100), which further comprises a step of introducing and expressing a *PDI* gene.

102. A process according to (101), wherein the *PDI* gene is a gene (M62815) from *Saccharomyces cerevisiae*.

103. A process according to (101) or (102), wherein the *PDI* gene is transferred into the vector of claim 83 and expressed.

104. A process according to any one of (94) to (103), which further comprises a step of introducing and expressing a heterologous gene.

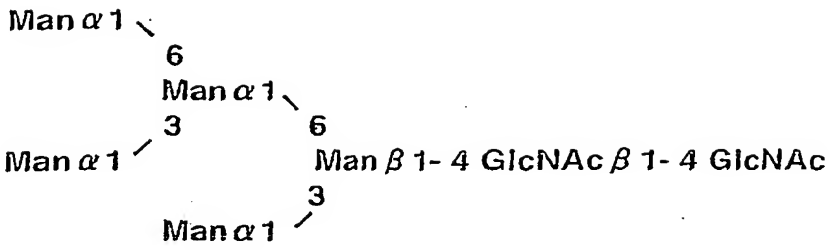
105. A process according to (104), wherein the heterologous gene is transferred into the vector of claim 83 and expressed.

106. A process for producing a protein encoded by a heterologous gene, which comprises culturing *Ogataea minuta* produced by the process of (104) or (105) in a medium, to obtain the protein comprising a mammalian type sugar chain encoded by the heterologous gene from the culture.

107. A protein comprising a mammalian type sugar chain encoded by a heterologous gene, wherein the protein has been produced by the process of (106).

108. A process for producing an *Ogataea minuta* strain, which is capable of producing a mammalian type sugar chain represented by the following structural formula ($Man_5GlcNAc_2$):

Structural Formula 2



wherein the process comprises the steps of:

disrupting an *OCH1* gene represented by SEQ ID NO:42 in an *Ogataea minuta* strain; and

disrupting a URA*3* gene represented by SEQ ID NO:15 in the same strain; and

disrupting a *PEP4* gene represented by SEQ ID NO:51 in the same strain; and

disrupting a *PRB1* gene represented by SEQ ID NO:57 in the same strain.

109. A process according to (108), wherein the *Ogataea minuta* strain is from the strain IFO 10746.

110. A process according to (108) or (109), which further comprises a step of disrupting an *ADE1* gene comprising the nucleotide sequence represented by SEQ ID NO:27.

111. A process according to (110), which further comprises a step of disrupting a *KTR1* gene comprising the nucleotide sequence represented by SEQ ID NO:63.

112. A process according to (111), which further comprises a step of disrupting an *HIS3* gene comprising the nucleotide sequence represented by SEQ ID NO:99.

113. A process according to (111), which further comprises a step of disrupting a *LEU2* gene comprising the nucleotide sequence represented by SEQ ID NO:107.

114. A process according to (111), which further comprises the step of:

1) disrupting a *YPS1* gene comprising the nucleotide sequence represented by SEQ ID NO:115.

115. A process according to any one of (108) to (114), which further comprises a step of introducing and expressing an α-1,2-mannosidase gene.

116. A process according to (115), wherein the α-1,2-mannosidase gene is transferred into the vector of (83) and expressed.

117. A process according to any one of claims 108 to 116, which further comprises a step of introducing and expressing a *PDI* gene (M62815).

118. A process according to (117), wherein the *PDI* gene (M62815) is transferred into the vector of (83) and expressed.

119. A process according to any one of (108) to (118), which further comprises a step of introducing and expressing a heterologous gene.

120. A process according to claim 119, wherein the heterologous gene is transferred into the vector of (83) and expressed.

121. A process for producing a protein encoded by a heterologous gene comprising a mammalian type sugar chain, wherein the process comprises culturing *Ogataea minuta* produced by the process of (119) or (120) in a medium to obtain the protein from the culture.

122. A protein encoded by a heterologous gene comprising a mammalian type sugar chain, wherein the protein has been produced by the process of (121).

This specification includes the contents disclosed by the specification and/or drawings of the Japanese Patent Application No. 2002- 127677, which is the basis of the priority claim of this application.

Brief Description of the Drawings

Fig. 1 shows the biosynthesis pathway of N-linked sugar chains, which is general in mammals.

Fig. 2 shows the biosynthesis pathway of N-linked sugar chains in yeast (*S. cerevisiae*), wherein M is mannose, and α2, α3, α6 and β4 mean α-1,2 linkage, α-1,3 linkage, α-1,6 linkage and β-1,4 linkage, respectively.

Fig. 3 shows the ^{1}H-NMR analysis of cell wall sugar chains of various yeasts.

Fig. 4 shows the HPLC (amide column) analysis of digests which were obtained by digesting sugar chains prepared from mannoproteins of cell walls of various yeasts by *Aspergillus saitoi* α-1,2-mannosidase (product of Seikagaku Corporation).

Fig. 5 shows the restriction maps of plasmids pOMGP1, pOMGP2, pOMGP3 and pOMGP4.

Fig. 6 shows the restriction maps of plasmids pOMUR1, pOMUM1 and pDOMU1.

Fig. 7 shows the structures of the *URA3* loci of a wild strain of *Ogataea minuta*, a strain transformed with plasmid pDOMU1 and a *URA3* gene knockout mutant, along with positions of PCR primers.

Fig. 8 shows the restriction maps of plasmids pOMAD1 and pDOMAD1. The restriction enzyme sites added artificially are underlined.

Fig. 9 shows the restriction maps of plasmids pOMUR2 and pROMU1.

Fig. 10 shows the structures of the *ADE1* loci of a wild strain of *Ogataea minuta*, an *ADE1* gene knockout mutant disrupted by plasmid pDOMAD1, and a *URA3* gene deficient mutant, along with positions of PCR primers.

Fig. 11 shows the restriction maps of plasmids pOMOC1, pOMOC2B, pOMOC3H and pDOMOCH1. The restriction enzyme sites of the vector are underlined.

Fig. 12 shows the structures of the *OCH1* gene loci of a wild strain of *Ogataea minuta*, an *OCH1* gene knockout mutant disrupted by the plasmid pDOMOCH1, and a *URA3* gene deficient mutant, along with positions of PCR primers.

Fig. 13 shows the structure analysis by an amide and reverse phase columns for sugar chains of the mannan glycoproteins of *Ogataea minuta* strain TK3-A which is an *OCH1* gene knockout mutant and of its parent strain *Ogataea minuta* strain TK1-3.

Fig. 14 shows the restriction maps of plasmids pOMPA1 and pDOMPA1, and the structures of the *PEP4* loci of a wild strain of *Ogataea minuta*, a *PEP4* gene knockout mutant disrupted by plasmid pDOMPA1, and a *URA3* gene deficient mutant. The restriction enzyme sites of the vector origin are underlined.

Fig. 15 shows the restriction maps of plasmids pOMPB1 and pDOMPB1, and the structures of the *PRB1* loci of a wild strain of *Ogataea minuta*, a PRB1 gene knockout mutant disrupted by plasmid pDOMPB1, and a *URA3* gene deficient mutant.

Fig. 16 the restriction maps of plasmids pOMKR1 and pDOMKR1, and the structures of the *KTR1* loci of a wild strain of *Ogataea minuta*, a *KTR1* gene knockout mutant disrupted by plasmid pDOMKR1, and a *URA3* gene deficient mutant. The restriction enzyme sites of the vector are underlined.

Fig. 17 shows the restriction maps of plasmids pOMMN9-1 and pDOMN9, and the structures of the *MNN9* loci of a wild strain of *Ogataea minuta*, an *MNN9* gene knockout mutant disrupted by the plasmid pDOMN9 and a *URA3* gene deficient mutant, along with positions of PCR primers.

Figs. 18 A and 18B show the restriction maps of plasmids pOMAX1, pOMAXPT1, pOMUR5, pOMUR6, pOMUR-X, pOMUR-XN, pOMex1U, pOMex2U, pOMex3G, pOMex4A, pOMex5H, pOMexGP1U and pOMexGP4A. The restriction enzyme sites of the vector are underlined.

Fig. 19 shows the structure analysis by amide and reverse phase columns for sugar chains of the mannan glycoprotein of *Ogataea minuta* strain TK3-A-MU1, which is an och1Δ strain expressing an *Aspergillus saitoi*-derived α-1,2-mannosidase gene.

Fig. 20 shows the structure analysis by amide and reverse phase columns of the *Saccharomyces cerevisiae*-derived invertase produced by *Ogataea minuta* strain TK3-A-MU-IVG1, which is an *Ogataea minuta OCH1* gene knockout mutant expressing *Aspergillus saitoi*-derived α-1,2-mannosidase gene.

Fig. 21 shows the Western analysis of the antibody produced by using *Ogataea minuta* strain TK9-IgB-aM.

Fig. 22 shows the purification of the antibody produced by using *Ogataea minuta* strain TK9-IgB-aM.

Fig. 23 shows the binding activity to G-CSF of the antibody produced by using *Ogataea minuta* strain TK9-IgB-aM.

Fig. 24 shows the analysis of the sugar chains of antibodies produced by using *Ogataea minuta* strain TK9-IgB and *Ogataea minuta* strain TK9-IgB-aM.

Fig. 25 shows the restriction maps of plasmids pOMHI1, pOMHI2, pOMHI3, pOMHI4 and pDOMHI1. The restriction enzyme sites of the vector and linker are underlined.

Fig. 26 shows the structures of the *HIS3* loci of a wild strain of *Ogataea minuta*, an *HIS3* gene knockout mutant disrupted by plasmid pDOMHI1, and a *URA3* gene deficient mutant, along with positions of PCR primers.

Fig. 27 shows the construction of plasmid pOMex6HS and its restriction map. The restriction enzyme sites of the vector and linker are underlined.

Fig. 28 shows the restriction maps of plasmids pOMLE1, pOMLE2 and pDOMLEl. The restriction enzyme sites of the vector and linker are underlined.

Fig. 29 shows the structures of the *LEU2* loci of a wild strain of *Ogataea minuta*, a *LEU2* gene knockout mutant disrupted by the plasmid pDOMLE1, and a *URA3* gene deficient mutant, along with positions of PCR primers.

Fig. 30 shows the construction of plasmid pOMex7L and its restriction map. The restriction enzyme sites of the vector and linker are underlined.

Fig. 31 shows the restriction maps of plasmids pOMYP1, pOMYP2, pOMYP3 and pDOMYP1. The restriction enzyme sites of the vector and linker are underlined.

Fig. 32 shows the structures of the *YPS1* loci of a wild strain of *Ogataea minuta*, a *YPS1* gene knockout mutant disrupted by plasmid pDOMLE1 and a *URA3* gene deficient mutant, along with positions of PCR primers.

Fig. 33 shows the Western analysis of the antibody produced by using *Ogataea minuta* strain YK3-IgB-aM.

Fig. 34 shows the purification of the antibody produced by using *Ogataea minuta* strain YK3-IgB-aM (Western analysis, and reducing & non-reducing condition).

Fig. 35 shows the Western analysis of the antibody produced by using *Ogataea minuta* strain YK3-IgB-aM-PDI.

Abbreviation

GlcNAc, GN: N- acetylglucosamine

Man, M : mannose

PA : 2- amino pyridylation

Modes for Carrying out the Invention

Hereinafter, the invention will be described in detail.

According to the invention, the process for producing a glycoprotein comprising a mammalian type sugar chain(s) comprises the following steps of:

1) breeding a methylotrophic yeast strain producing a mammalian type sugar chain, by introducing an α-1,2-mannosidase gene into a mutant strain comprising mutated sugar chain biosynthesizing enzyme genes (for example, an *OCH1* gene (α-1,6-mannosyl transferase) knockout mutant, which is considered to be a key enzyme for the elongation reaction where mannose residues attach to an M8 high mannose type sugar chain one by one via α-1,6 linkage), and expressing it under the control of a potent promoter such as methanol-inducible promoter; and

2) culturing in a medium the yeast strain bred by introducing heterologous genes into a mutant yeast which comprises mutated sugar chain biosynthesizing enzyme genes and expressing these genes, and obtaining the glycoproteins comprising a mammalian sugar chain from the culture.

1. Preparation of mammalian type sugar chain producing strains

According to the present invention, mutant strains of yeast capable of producing mammalian type sugar chains, wherein the mutant strain has a disruption in its outer chain biosynthesis gene specific to yeast and has been deprived of sugar chains specific to yeast, can be prepared in the following manner.

1-1 Preparation of Man5 type sugar chain ("high mannose type sugar chain") producing yeasts

Mutation trait necessary for the mutant yeast of the invention is a mutation of a gene(s) peculiar to yeast associated with the outer sugar chain biosynthesis system, and specifically at

least a mutation of *OCH1* gene. That is, as long as the mutant yeast has the above-mentioned mutation, it may be either a natural mutant strain or an artificial mutant strain.

The *OCH1* gene means a gene encoding α-1,6 mannosyl transferase, which catalyses the initial reaction of the outer sugar chain formation in yeast, and works to further transfer a mannose residue to the core sugar chain of N-linked sugar chain of a glycoprotein of yeast via α-1,6-linkage. This reaction functions as a trigger for attaching mannose excessively compared with the glycoproteins of animal cells ("hyper-mannosylation"), thereby forming a mannan-type sugar chain peculiar to yeast. Therefore, *OCH1* gene encodes a protein having the above-mentioned activity and function strictly, and it does not refer to a gene which simply has a homology to the gene sequence or the amino acid sequence deduced from the gene sequence.

However, in order to change the sugar chain of yeast into a mammalian type sugar chain, just the manipulation that disrupts this *OCH1* gene is not enough.

As mentioned above, in a mammalian cell, α-mannosidase I acts on a high mannose type sugar chain to cut off several mannose residues, and finally generates a Man5 high mannose type sugar chain ("Man5GlcNAc2"). This Man5 type sugar chain serves as a prototype of mammalian type sugar chain. N-acetylglucosaminyl transferase (GnT) I acts on this sugar chain, and causes the transfer of one N-acetylglucosamine residue to generate a hybrid type sugar chain which comprises GlcNAcMan5GlcNAc2, followed by successive formation of complex type sugar chains. Therefore, to make a yeast cell to produce a mammalian type sugar chain(s), it would be necessary to create a yeast which produces a Man5 high mannose type sugar chain (i.e., Man5GlcNAc2) first.

α-1,2-mannosidase (also referred to as α-mannosidase-I) as used in the invention is not limited as long as it has the above-mentioned enzyme activity. For example, α-mannosidase-I involved in the above-mentioned sugar chain biosynthesis system in mammalian cells, α-mannosidase enzymes from other animals such as nematode, and α-1,2-mannosidase enzymes from fungi such as *Aspergillus saitoi* can be used.

In order to effect the invention efficiently, the expression site of α-1,2-mannosidase is important. It is said that α-1,2-mannosidase functions in the *cis* Golgi in mammalian cells.

On the other hand, addition of a sugar chain peculiar to yeast in the yeast cell is performed in the *cis*, *medial* or *trans* Golgi. Therefore, it is necessary to make α-1,2-mannosidase act prior to the modification in which a sugar chain peculiar to yeast is attached, i.e., modification in Golgi apparatus. If the expression site is in the Golgi apparatus which exists downstream in the transportation pathway of glycoprotein, then Man5 type sugar chains cannot be generated efficiently.

Therefore, to attain this purpose, endoplasmic reticulum (ER) retention signal (for example, amino acid sequence shown by His-Asp-Glu-Leu) in yeast may be attached to the C terminus of the protein of α-1,2-mannosidase thereby localizing the enzyme within ER to cause expression of the activity so that the attachment of sugar chain peculiar to yeast can be inhibited. This method was already reported by inventors (Chiba et al., J.Biol.Chem., 273, 26298-26304 (1998)).

However, when the sugar chain of a certain protein is changed into a mammalian type sugar chain in order to use this protein as a drug, it is required to remove sugar chains peculiar to yeast almost completely, and use of only the above-mentioned technique is supposed to be insufficient. In fact, although in the above-mentioned report Chiba et al. use the promoter of glyceraldehyde-3-phosphate dehydrogenase, which is known to be the strongest promoter functioning in *Saccharomyces cerevisiae*, in the expression of the glyceraldehyde-3-phosphate dehydrogenase, the results of analyzing the sugar chains of cell wall glycoproteins reveal that Man5 type sugar chains were generated in the level of only about 10%.

The system using the sugar chain mutant of *Ogataea minuta* in the invention enables formation of a Man5 type sugar chain in the amount of 20% or more, preferably 40% or more, more preferably 60% or more, most preferably 80% or more of the sugar chains of the cell wall glycoproteins which the yeast produces as in the Examples below. Also, Man5 type sugar chains are formed in the amount of 20% or more, preferably 40% or more, more preferably 60% or more, most preferably 80% or more in the example of the secretion and expression of a heterologous gene. Thus the problems in *Saccharomyces cerevisiae* have been solved. The application of *Ogataea minuta* in the invention to various glycoproteins will be expected from these results.

On the other hand, Chiba et al. uses the Δoch1Δmnn1Δmnn4 strain which generates only the Man8 type sugar chain, a core sugar chain. *MNN1* gene is presumed to be a gene peculiar to *Saccharomyces cerevisiae*, and the sugar chain synthesis pathway and sugar chain synthesizing genes was isolated and analyzed, but sugar chain structure was not fully analyzed for other yeasts. For example, the existence of a sugar chain which has β- mannoside linkage is known for *Pichia pastoris* as mentioned above (Higgins (ed.), Pichia Protocols, 1998, pp. 95-105, Humana Press and Biochim.et Biophy. Acta, 1426, 227-237 (1999)). Moreover, the results of SDS-PAGE of the glycoproteins produced by the *OCH1* gene homologue knockout mutant disclosed in Japanese Patent Publication (Kokai) No. 9-3097A (1997) surely presented the data indicating that the sugar chains have been shortened into lower molecules; namely, it is presumed that they are not glycoproteins having a single sugar chain like Man8 type sugar chain. No gene involved in the synthesis of these sugar chains has been isolated, and great labors are needed for isolating and disrupting the gene.

Thus, to allow a yeast strain to produce Man5 type sugar chains, it is necessary to cause α-1,2-mannosidase to highly express, and for this purpose, a potent promoter is needed. In these circumstances, the invention was completed by using an alcohol oxidase (*AOX*) gene promoter (inducible by methanol) from methylotrophic yeast known as the strongest inducible expression promoter. Other inducible expression promoters usable in the invention include, but not limited to, promoters for dihydroxyacetone synthetase (*DAS*) gene and formate dehydrogenase (*FDH*) gene, and any promoter can used as long as it has an ability to express the enzyme gene in the methylotrophic yeast of the invention.

Thus, mammalian type sugar chains can be produced without disrupting an outer sugar chain synthesis gene peculiar to yeast, by preliminarily trimming (removing) the sites on the sugar chain to which sugar chains peculiar to yeast is attached in the ER and Golgi apparatus. Accordingly, the acquisition of a gene for forming β-mannoside linkage and of an *MNN4* gene, which is for addition of mannose phosphate, becomes unnecessary.

However, *OCH1* exists quite ubiquitously in yeast, and the location thereof is relatively near the reducing terminal side of the core sugar chain and so it is believed that the gene should be destroyed in order to remove its activity.

Yeast strains applicable to the invention include any strain in which the sugar chain of glycoprotein mainly comprises α-1,2-mannoside linkage, and methylotrophic yeasts are not limited as long as they produce N-linked sugar chains which mainly comprise α-1,2-mannoside linkage, including as specific examples *Ogataea minuta*, *Candida succiphila*, *Pichia pastoris*, *Pichia trehalophila*, *Pichia methanolica*, *Pichia angusta*, *Hansenulla polymorpha*, etc. Preferred is *Ogataea minuta*.

Therefore, the procedures disclosed by the invention are inapplicable to yeast strains having the structure where sugar chains other than α-1,6 mannose have been attached directly to the core sugar chain by the *OCH1* gene. That is, any yeast strain which generates glycoproteins with sugar chains peculiar to yeast attached to moieties of the core sugar chain, strictly to moieties of the Man5 type sugar chain, cannot utilize in the procedures of the invention.

Furthermore, mammalization can be more efficiently attained by auxiliary disruption of a *KTR* gene homologue belonging to α-mannosyl transferase gene family (for example, *KTR1* gene of *Ogataea minuta* as found in the invention), or of an *MNN9* gene homologue (for example, *MNN9* gene of *Ogataea minuta* as found by the invention) which is believed to be involved in the attachment of sugar chains in the Golgi apparatus.

Furthermore, since sugar chain mutants have generally shorter sugar chains in glycoproteins, and as a result, the cell wall becomes weaker, so the drug susceptibility increases or the resistance to osmotic pressure decreases in the mutants. In such a case problems may occur in cell culture. On the contrary, in the procedure of the invention, which utilizes a methanol-inducible promoter and expresses α-1,2-mannosidase, mammalian type sugar chains can be produced as a by-product along with a glycoprotein encoded by a heterologous gene. Hence, the culture and production can be performed without applying a burden at the time of multiplication of the yeast cell.

The term "a gene(s) associated with the mammalian type sugar chain biosynthesis" as described above means an appropriate number of transgenes, which belong to a group of one or more of the above-mentioned genes, required to produce a sugar chain of interest. When

the transgenes are plural, they may belong to a group of homo-type genes or to a group of hetero-type genes.

In order to obtain the produced sugar chains and glycoproteins in high yield, it is desirable to make the above-mentioned enzymes to express highly in a suitable organ (for example, Golgi apparatus). Therefore, it is effective to use genes compatible to the codon usage of yeast. Also, to localize the enzymes in a suitable organ, the addition of a signal sequence or the like of yeast will become effective. For the transfer of a gene, use of vectors such as chromosome integration type (YIp type) vector may be considered. Promoters required to express the gene include, but are not limited to, constitutive expression promoters such as GAPDH and PGK, inducible expression promoters such as AOX1, etc. However, since multiplication of yeast may be affected when one or more glycosidase, glycosyltransferases, or sugar nucleotide transporter genes are expressed, it is necessary to take into consideration the use of an inducible promoter or the appropriate order of introducing genes.

The mutant yeast which produces the above-mentioned mammalian type sugar chain, or the mutant to which the above-mentioned foreign gene has been transferred, is cultured in a culture medium, thereby to produce glycoproteins comprising the same Asn-linked sugar chain as the high-mannose type sugar chain ($Man_5GlcNAc_2$), the hybrid type sugar chain ($GlcNAcMan_5GlcNAc_2$) or the complex type sugar chain (for example, $Gal_2GlcNAc_2Man_3GlcNAc_2$), which the mammalian cell produces, either intracellularly or extracellularly. In this case, the content of an outer sugar chain peculiar to yeast is significantly reduced.

Specifically, the transfer of a GnT-I gene into the above-mentioned mutant enables production of a hybrid type sugar chain, and the transfer of a gene(s) associated with the mammalian type sugar chain biosynthesis system (α-mannosidase II, GnT-II, GalT, UDP-GlcNAc Transporter, and /or UDP-Gal Transporter genes) enables production of a double-stranded complex type sugar chain ($Gal_2GlcNAc_2Man_2GlcNAc_2$).

2. Different genes from *Ogataea minuta* usable in the invention

The proteins usable in the invention are not particularly limited as long as they have respective activities, and specifically they are proteins comprising an amino acid sequence substantially represented by the SEQ ID NO described in the Examples below. As used herein, the term "an amino acid sequence substantially represented by SEQ ID NO:X" means that the amino acid sequence includes:

(a) the amino acid sequence represented by SEQ ID NO:X; or

(b) an amino acid sequence which comprises a deletion(s), a substitution(s) or an addition(s) of one or several amino acids in the amino acid sequence represented by SEQ ID NO:X.

That is, the above amino acid sequence may be partially modified (for example, substitution, deletion, insertion or addition of an amino acid residue(s) or a peptide chain(s), etc.). Herein, the term "several" in relation to the number of deleted, substituted or added amino acids means any number in the range capable of being introduced by the methods usually used in art, preferably 2 to 10, more preferably 2 to 5, and most preferably 2 to 3.

DNAs comprising a nucleotide sequence, which encodes the protein usable in the invention, are characterized by comprising the nucleotide sequences encoding the above-mentioned proteins from *Ogataea minuta* as defined in the invention. Such nucleotide sequences are not particularly limited as long as they are the nucleotide sequences encoding the proteins of the invention, and their examples are the nucleotide sequences which encode amino acid sequences substantially represented by the SEQ ID NOs described in the Examples below. As used herein, the term "a nucleotide sequence substantially represented by SEQ ID NO:X" means that the nucleotide sequence includes:

(a) the nucleotide sequence represented by SEQ ID NO:X; or

(b) a nucleotide sequence comprising a deletion, a substitution or an addition of one or several nucleotides in the nucleotide sequence represented by SEQ ID NO:X.

This DNA may be conventionally produced by the known procedures. For example, all or part of the DNA may be synthesized by using a DNA synthesizer based on the nucleotide sequence illustrated in the invention, or may be prepared by PCR amplification using chromosome DNA. Here, the term "several" in relation to the number of deleted,

substituted or added nucleotides means any number in the range capable of being introduced by the methods usually used in art, for example, site-directed mutagenesis (e.g., Molecular Cloning, A Laboratory Manual, second edition, ed. by Sambrook et al., Cold Spring Harbor Laboratory Press, 1989; CurrentProtocols in Molecular Biology, John Wiley & Sons (1987-1997)), for example, 2 to 10, preferably 2 to 5, and more preferably 2 to 3.

3. Obtaining genes

Isolation of a target gene fragment can be performed by extracting genomic DNA from a yeast strain, and selecting the target gene, by using general procedures (Molecular Cloning (1989), Methods in Enzymology 194 (1991)). In the above, the genomic DNA from *Ogataea minuta* can be extracted, for example, by the methods of Cryer et al. (Methods in Cell Biology, 12, 39-44 (1975)) and of P. Philippsen et al. (Methods Enzymol., 194, 169-182 (1991)).

For example, the protoplast prepared from yeast can be subjected to a conventional DNA extraction method, an alcohol precipitation method after removing cell debris under high salt concentration, an alcohol precipitation method after extracting with phenol and/or chloroform, etc. Besides the above method utilizing the preparation of protoplast, DNA may be extracted by break of cells with glass beads. The protoplast method is preferable because preparation of high molecular weight DNA is easy.

A target gene can be obtained, for example, by the PCR method (PCR Technology, Henry A. Erlich, Atockton press (1989)). The PCR is a technique which enables *in vitro* amplification of a specific DNA fragment to hundreds of thousands fold or more in about 2 to 3 hours, using a combination of sense/antisense primers annealed at each end of the target region, a heat-resistant DNA polymerase, and a DNA amplification system. In the amplification of a target gene, 25-30mer synthetic single-stranded DNAs and genomic DNA can be used as primers and as a template, respectively. The amplified gene may be identified in terms of its nucleotide sequence before use.

The DNA sequence of a gene can be determined by usual methods such as, for example, dideoxy method (Sanger et al., Proc. Natl. Acad. Sci., USA, 74, 5463-5467 (1977)).

Alternatively, the nucleotide sequence of DNA can easily be determined by use of commercially available sequencing kits or the like.

The isolation, purification, etc. of the DNA can also be carried out by ordinary methods, and in the case of *E. coli* for example, the DNA may be extracted by the alkali/SDS method and ethanol precipitation, and the DNA subsequently purified by RNase treatment, PEG precipitation or the like.

A target gene can also be obtained by: (a) extracting the total DNA of the above-mentioned yeast, transferring a gene transfer vector, which comprises a DNA fragment derived from said DNA, into a host, thereby to prepare a gene library of the yeast, and (b) subsequently selecting the desired clone from the gene library, followed by amplifying the clone.

The gene library can be prepared as a genomic library by partially digesting the chromosomal DNA obtained by the above-mentioned method with appropriate restriction enzymes (such as Sau3AI) to obtain fragments thereof, ligating the fragments with an appropriate vector, and transforming an appropriate host with the vector. Alternatively, it is also possible by amplifying a fragment of the target gene by PCR first, screening for restriction sites by the genomic Southern analysis so that the target gene can be obtained efficiently, and digesting the chromosomal DNA by this restriction enzyme to obtain the desired fragment. Vectors usable for this purpose include commercially available plasmids such as pBR system, pUC system, Bluescript system, etc., usually known as the known vectors for preparing a gene library. Phage vectors of Charon system or EMBL system etc. or cosmids can be also used widely. The host to be transformed or transduced with the prepared vector for preparation of gene library can be selected depending on the type of the above-mentioned vectors.

Clones can be selected and obtained from the above-mentioned gene library using a labeled probe which comprises a sequence peculiar to a target gene, by means of colony hybridization, plaque hybridization or the like. A sequence peculiar to target gene used as a probe can be obtained by synthesizing a corresponding oligonucleotide of the gene which encodes the amino acid sequence of a target protein purified from *Ogataea minuta*,

specifically amplifying the desired DNA fragment by PCR using the chromosomal DNA of *Ogataea minuta* as a template, to obtain it. The peculiar sequence may also be obtained by searching for a gene which encodes a protein homolog from different species in DNA databases such as GenBank or protein databases such as SWISS-PROT, to obtain the sequence information, synthesizing an oligonucleotide corresponding to the conserved amino acid sequence analyzed with an analyzing software such as homology search programs such as BLAST, GENETYX (Software Development), and DNAsis (Hitachi Software), and specifically amplifying the desired DNA fragment by PCR using the chromosomal DNA of *Ogataea minuta* as a template. The synthesized oligonucleotide may be used as a probe. Once the nucleotide sequence is determined, the desired gene can be obtained by chemical synthesis or PCR using primers synthesized based on the determined nucleotide sequence, or by hybridization using as a probe the DNA fragment comprising the above-mentioned nucleotide sequence.

4. Gene disruption

In the invention, a target gene is basically disrupted in accordance with the method disclosed by Rothstein, in Methods Enzymol., 101, 202-211 (1983). Specifically, a target gene DNA obtained by the above-described method is first cut or partially deleted, an appropriate selectable marker gene DNA is inserted at the cut or deleted site, thereby to prepare a DNA structure in which the selectable marker has been sandwiched between upstream and downstream regions of the target gene. Subsequently, this structure is transferred to a yeast cell. The above manipulation results in two recombinations at homologous moieties between each end of the transferred fragment (i.e., the DNA structure with a selectable marker sandwiched) and a target gene on chromosome, thereby substituting the target gene on chromosome with the transferred fragment. Auxotrophic markers and drug resistant markers, as shown below, may be used as the selectable marker for gene disruption. In this case, one selectable marker will generally be required for disrupting one gene. When *URA3* gene is used, ura3 trait can be efficiently reproduced and so it is often used for this purpose.

Specific explanation is provided using an example of the preparation of an *OCH1* gene knockout strain. A plasmid carrying *URA3* gene, which comprises a repeated structure before and after structural gene, is constructed, and the gene cassette cleaved out with a restriction enzyme is inserted at a target gene on the plasmid, thereby to construct a disrupted allele. Gene-knockout strain can be obtained by substituting with a target gene on the chromosome using this plasmid. As the *URA3* gene inserted into the chromosome is sandwiched by the repeated structures, it is dropped out of the chromosome due to homologous recombination between the repeated structures. The selection of this *URA3* deficient strain can be carried out by use of 5-fluoroorotic acid (5-FOA). A *ura3* mutant is resistant to 5-FOA (Boeke et al., Mol. Gen. Genet., 197, 345-346 (1984); Boeke et al., Methods Enzymol.,154, 165-174 (1987)), and a cell strain having URA3+ phenotype can no longer grow in the 5-FOA medium. Thus, separating a strain with resistant trait in a medium to which 5-FOA is supplemented, enables manipulations using a *URA3* gene marker again. Therefore, the mutated auxotrophic trait of the original yeast strain is not damaged by gene destruction in the "artificial knockout mutant" which has undergone the gene disruption artificially by this technique.

In addition, in the "natural mutant" where the gene disruption occurs naturally without using the above-mentioned procedures but spontaneously, the number of the mutated auxotrophic traits is not decreased nor increased.

5. Marker for gene transfer

The auxotrophic marker for transfer of a heterologous gene into the mutant yeast of the invention is defined by yeast strains to be used, and is specifically selected from *ura3*, *his3*, *leu2*, *ade1* and *trp1* mutations. Although the number of auxotrophic markers depends on the number of transfer genes, generally one auxotrophic marker is required for transfer of one gene. When plural of genes are transferred, a larger number of auxotrophic markers become necessary as the number of transfer genes increases more and more, since the transfer gene fragment is longer, and transfer efficiency decreases, and as a result, expression efficiency also decreases.

In the invention, the gene which complements the auxotrophy is a gene associated with the *in vivo* synthetic system of biological components such as amino acids and nucleic acids. The complementing gene is an original functional gene itself, since the mutated traits include such a mutation that the gene fails to function. Therefore, the gene from the original yeast strain is desirable.

Usable selectable markers other than the above-mentioned auxotrophic markers include drug resistance markers, which impart resistance to drugs such as G418, cerulenin, aureobasidin, zeocin, canavanine, cycloheximide, hygromycin and blastcidin, and may be used to transfer and disrupt a gene. Also, it is possible to perform the transfer and disruption of a gene by using, as a marker, the gene which imparts a solvent resistance like ethanol resistance, an osmotic pressure resistance like resistance to salt or glycerol, and a metal ion resistance like resistance to copper, etc.

6. Method for transfer of DNA into cell and transformation with same

Methods for transferring a DNA into a cell for its transformation with the DNA in the above procedures include general methods, for example, a method of incorporating a plasmid into a cell after the cell is treated with lithium salt so that the DNA is prone to be naturally transferred into the cell (Ito et al., Agric. Biol. Chem., 48, 341 (1984)), or a method of electrically transferring a DNA into a cell, a protoplast method (Creggh et al., Mol. Cell. Biol., 5, 3376 (1985)), and the like (Becker and Guarente, Methods Enzymol., 194, 182 -187 (1991)). The expression vector of the invention can be incorporated into the host chromosome DNA, and can exist stably.

7. Expression of heterologous gene

The term "heterologous gene" as used herein is a gene of interest to be expressed, and means any gene different from the gene for *Ogataea minuta*-derived alcohol oxidase or glyceraldehyde-3-phosphate dehydrogenase. Examples of heterologous genes include: enzyme genes such as acidic phosphatase gene, α-amylase gene and α-galactosidase gene; interferon genes such as interferon α gene and interferon γ gene; interleukin (IL) genes such as

IL1 and IL2; cytokine genes such as erythropoietin (EPO) gene and granulocyte colony stimulating factor (G-CSF) gene; growth factor genes; and antibody genes. These genes may be obtained by any procedures.

To utilize the invention efficiently, a gene encoding a glycoprotein produced by a mammal cell, particularly human cell, can be used. That is, since the object of the invention is to produce a glycoprotein which has the same or similar sugar chain structure as that of mammals particularly human, the invention is effectively applied to the glycoprotein which has a sugar chain structure on the protein molecule, and additionally to useful physiologically active proteins including antibodies. An antibody has been used as a medicament for many years. The antibody, however, was from an origin other than a human and so it causes the production of an antibody against the administered antibody itself. Accordingly, multiple administrations cannot be conducted, so its use is limited. In recent years, humanized antibody in which the amino acid sequence except the antigen-binding site is replaced by a sequence of human antibody, has been prepared. Furthermore, a mouse producing human antibody into which human antibody gene has been transferred has been created. Complete human antibody is now available and the use of an antibody as drug has prevailed quickly. These antibodies can be produced by hybridomas or by cultured cells such as CHO cell, which comprise a transfer gene encoding an antibody, however there are many problems in respect of productivity, safety, etc. Under such a circumstance, production of antibodies using yeast is expected, because the above problems may be overcome by the use of yeast. In this case, as the antibody molecule is a glycoprotein to which N-type sugar chains are attached at two or more sites in each heavy chain, and when the antibody is produced with yeast, sugar chains peculiar to yeast are attached thereto. These sugar chains have antigenicity by themselves as mentioned above, and/or an action to decrease physiological activity. Hence, when the antibody produced with yeast is used as a medicament, the conversion of the sugar chain to a mammalian type is unavoidable.

In the meantime, the method for preparing antibodies with high ADCC activity has been reported, which method comprises removal of α-1,6-fucose attached to GlcNAc on the side of the reduced terminus of a sugar chain (PCT/JP00/02260). Although α-1,6-fucosyl

transferase gene (*FUT8*) is known as a gene involved in addition of α-1,6-fucose, this gene is present ubiquitously in animal cells, and unless the cells deficient in this enzyme activity or the cells in which this gene is artificially disrupted are used, part of the prepared antibody is inevitably attached with α-1,6-fucose.

On the contrary, since the yeast generally has no synthetic systems of fucose and α-1,6-fucosyl transferase gene (*FUT8*), glycoproteins free from α-1,6-fucose can be produced without artificial gene disruption. So, highly active antibodies could be naturally produced.

While there is a report on high production of antibody fragments such as Fab and ScFv in yeast, there is almost no report on high production of a full-length antibody. Since antibody fragments such as Fab and ScFv do not comprise the Fc domain which exists in the heavy chain of an antibody, they have neither antibody-dependent cellular cytotoxicity (ADCC) nor complement-dependent cytotoxicity (CDC), which is a physiological activity peculiar to an antibody, and their use as drug is restricted. The antibody has 14 disulfide (S-S) linkages in total, and it is presumed that the reason why full-length antibody cannot be highly produce within a yeast cell is due to that the antibody molecule cannot appropriately fold. Although this cause is not clear, it cannot be denied that the phenomenon may possibly be caused by difference in the structure of N-type sugar chain attached to the antibody heavy chain. So, use of the yeast of the invention producing mammalian sugar chains may enable the efficient production of an antibody molecule having suitable conformation. Probably, functional antibody may also be highly produced by introducing Protein Disulfide Isomerase (PDI), a molecule chaperon. In addition, according to the invention, it is possible to produce either an intact antibody molecule or other antibody fragments as mentioned above, or other antibody fragments as long as it has a desired function. The antibody is not particularly limited, but preferred antibody includes a humanized antibody in which an antibody-binding site of another mammalian antibody is introduced into a mammalian, particularly preferably human type framework, or a human antibody. Although not limited particularly, the antibody to be expressed is preferably in the class of IgG and more preferably in the subclass of IgG1.

When a heterologous protein is produced by the gene recombinant technology, it is sometimes degraded by a protease in the host. In such a case, the production of the protein of interest decreases, heterogeneous proteins generate, and the purification of the protein becomes difficult due to the contamination of proteolysis products.

In order to circumvent these problems, such a culture method that the activity of a protease degrading the desired protein is inhibited has been studied, for example, a method of adjusting the pH of a medium for culturing a recombinant cell to inhibit a protease activity. However, this method will affect the growth of host yeast which expresses a certain type of heterologous protein, and is effective only for the degradation of the protein outside the cell.

There is an example which increased the production of cell proteins present inside and outside the cell by using a protease deficient strain in which proteinase A and proteinase B have been inactivated in *Saccharomyces cerevisiae*, *Pichia pastoris*, or *Candida boidinii* (Japanese Patent Publication (Kohyo) No.6-506117A (1994), Weis, H.M. et al., FEBS Lett., 377, 451 (1995), Inoue, K. et al., Plant Cell Physiol., 38 (3), 366 (1997), and Japanese Patent Publication (Kokai) No.2000-78978).

Proteinase A and proteinase B are proteases located in the vacuole and are encoded by *PEP4* gene and *PRB1* gene, respectively. According to researches on yeast *Saccharomyces cerevisiae*, proteinase A and proteinase B activate themselves and other proteases such as carboxypeptidase Y (vandenHazel, H.B. et al., YEAST, 12, 1 (1996)).

In the meantime, Yapsin is a protease which exists widely in the Golgi apparatus and cell membrane, and according to researches on *Saccharomyces cerevisiae*, it was isolated as a homologue of the protein encoded by *KEX2* gene known as a processing enzyme of α-factor. To date, genes of Yapsin1 (Aspartic proteinase 3, YAP3), Yapsin2 (Aspartic proteinase MKC7), Yapsin3, Yapsin6, Yapsin7, etc. are known (Egel-Mitani, M. et al., Yeast 6 (2), 127-137 (1990); Komano, H. et al., Proc. Natl. Acad. Sci. U.S.A. 92(23), 10752-10756 (1995); and Saccharomyces Genome Database (SGD)). Of them, Yapsin1 is encoded by YPS1 gene.

An example in which the production of cell proteins present inside and outside the cell was increased by using a protease deficient *Saccharomyces cerevisiae* strain in which Yapsin1

has been inactivated is known (M. Egel-Mitani et al., Enzyme and Microbial Technology, 26, 671 (2000); Bourbonniais, Y. et al., Protein Expr. Purif., 20, 485 (2000)).

Ogataea minuta strains of the invention deficient in *PEP4* gene, *PEP4PRB1* gene or *PEP4PRB1YPS1* gene, whose protease activities have been reduced, maintain an ability to grow themselves equivalent to the wild strain under culture conditions of using a nutrition medium, and are thus very good hosts for the production of heterologous proteins. Therefore, the above-mentioned yeasts can efficiently produce heterologous proteins, such as an antibody highly susceptible to protease, due to suppressing the degradation of the yeasts.

8. Construction of expression cassette for heterologous gene

The expression system useful for production of proteins can be prepared by various methods. A protein expression vector comprises at least a promoter area, a DNA encoding the protein, and the transcription terminator area in the direction of the reading frame of transcription. These DNAs are arranged as related operably to each other so that the DNA encoding the desired glycoprotein may be transcribed to RNA.

The high expression promoter which can be used in the invention is preferably a methanol-inducible expression promoter, and includes, for example, alcohol oxidase (*AOX*) gene promoter of *Ogataea minuta*, dihydroxyacetone synthase (*DAS*) gene promoter of *Ogataea minuta*, formate dehydrogenase (*FDH*) gene promoter of *Ogataea minuta*, etc.

The constitutive expression promoter includes glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene promoter of *Ogataea minuta*, phosphoglycerokinase (*PGK*) gene promoter of *Ogataea minuta*, etc.

The transcription terminator may be the sequence that has an activity to cause the termination of the transcription directed by the promoter, and may be identical to or different from the promoter gene.

According to one aspect of the invention, we (1) obtained the nucleotide sequences of an *Ogataea minuta* alcohol oxidase(*AOX*) gene as a methanol-inducible expression cassette and a glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene as a constitutive expression cassette, along with their promoters and terminators, (2) isolated the promoters and terminators,

(3) constructed expression vectors, and (4) used the expression vectors of the invention to prepare transformed cells, and confirmed that when expressed in the transformed cells, heterologous genes are expressed in the same manner as the genes from *Ogataea minuta*. The expression cassette of a heterologous gene using the promoter and terminator for the alcohol oxidase (*AOX*) gene will be described below as an example.

8-1 Cloning of alcohol oxidase (*AOX*) gene

In order to obtain the expression cassette of the invention, alcohol oxidase (*AOX*) gene was cloned at first. As the starting material, yeast such as *Ogataea minuta* strain IFO 10746 is exemplified. Cloning of the gene can be performed by a method as mentioned above.

8-2 Isolation of promoter and terminator areas

Promoter and terminator areas can be cut out with a restriction enzyme(s) but generally a convenient restriction site does not necessarily exist at a suitable position. Accordingly, the nucleotide sequence may be cleaved in order from restriction sites in the coding area toward the promoter area by an endonuclease, thereby to find a clone deleted until the suitable position. Recently, a primer with a restriction enzyme recognition site at the end has been used to be easily able to amplify and obtain desired promoter and terminator areas by PCR.

It is also possible to chemically synthesize those areas, or alternatively, to make semi-synthesized promoter and terminator by use of both a DNA whose partial area is chemically synthesized and which is then cloned using the partial DNA, and a restriction enzyme site(s).

The sequence comprising a promoter area or a terminator area is illustrated in SEQ ID NO:79 or in SEQ ID NO:80, respectively. They are, however, not to be limited to the specific sequences, and the nucleotide sequences thereof may be modified by deletion, insertion, substitution, addition, or the like, as long as they essentially hold transcription activity.

Modification of the nucleotide sequences can be performed by any known mutagenesis method (e.g., by the method using TAKARALA LA PCR in vitro Mutagenesis kit, TAKARA

SHUZO CO., LTD., Japan), or the like. When the promoter area is deleted widely, this deletion may appropriately be conducted by PCR using a commercially available kit for deletion (e.g., Deletion kit for kilo sequences of TAKARA SHUZO CO., LTD.).

8-3 Construction of expression vector

The expression vector of the invention can be obtained by inserting *AOX* promoter, a heterologous structural gene, an *AOX* terminator, a marker gene and a homologous area into an appropriate vector. Examples of the vector used for this purpose include, but are not limited to, *E. coli* plasmid vectors such as the above-mentioned pBR system, pUC system and Bluescript system. Inserting the components of the expression vector into a vector can easily be carried out by those skilled in the art with reference to the description of Examples as described below or by conventional techniques. Those skilled in the art can determine the selectable marker gene and the homologous area easily. Examples of the marker gene include antibiotic resistance genes such as the above-mentioned G-418 and hygromycin resistant genes, and auxotrophy complementing genes such as *URA3*, *ADE1* (phosphoribosyl-amino-imidazole succinocarboxamide synthase), *HIS3* (imidazole-glycerol-phosphate dehydratase), *LEU2* (3-isopropylmalate dehydrogenase) genes.

DNA encoding a secretion signal sequence which functions in a yeast cell may be added to a heterologous structural gene. Since this expression system allows production and secretion of a glycoprotein out of the host cell, the desired glycoprotein can easily be isolated and purified. The secretion signal sequence includes secretion signal sequences of *Saccharomyces cerevisiae* α-mating factor (α-MF), *Saccharomyces cerevisiae* invertase (*SUC2*), human α-galactosidase, human antibody light chains, etc.

The constructed expression vector is a chromosome integration type vector, and the desired gene is incorporated by being integrated onto the chromosome. In the case of an auxotrophic marker type vector, a part of the marker gene is cleaved by a restriction enzyme(s) to form a single stranded marker gene. Then the transformation is performed and the vector is generally integrated into a part of the allele on the chromosome. In the case of a drug

resistance marker, no allele exists, and so the expression promoter or terminator area is cleaved by a restriction enzyme(s) to form a single stranded promoter or terminator. Then the transformation is performed and the vector is generally integrated onto the above-mentioned part on the chromosome. Once the gene is integrated, it exists on a chromosome, and maintained stably.

8-4 Use of expression vector

The expression vector using the *AOX* promoter of the invention is effective not only for expression of α-1,2-mannosidase gene and heterologous genes of interest but also for expression of other genes. By using expression vectors to which different types of selectable markers have been attached, the vectors can be transferred sequentially into a yeast cell, and high expression of plural genes can be achieved.

For example, the yeast is not a host which originally generates a significant amount of secreted proteins, when compared with mold or the like. Thus, it is expected that the yeast bears no complete secretion mechanism. In fact, as mentioned above, the productivity of an antibody in yeast is originally low.

Therefore, in order to enhance secretion efficiency, it is effective that a molecule chaperon or the like is introduced to attain high expression.

9. Production of glycoprotein having mammalian type sugar chain

To produce glycoproteins having the above-mentioned sugar chains from a heterogeneous organism, the above-mentioned yeast mutant strain is used as a host , and a gene in which a heterologous gene (e.g., cDNA) is ligated downstream of a promoter and can be expressed in the above-mentioned yeast, is prepared. The gene is integrated into the above-mentioned yeast host by homologous recombination or inserted into a plasmid to carry out transformation of the above-mentioned host. The thus prepared transformant of the above-mentioned host is cultured by known methods. The glycoprotein, which is encoded by the heterologous gene, produced intracellularly or extracelluraly is collected and purified, thereby obtaining the glycoprotein.

The above-mentioned mammalian type sugar chain producing yeast mutant strain maintains an ability to grow itself almost equivalent to the wild yeast strain, and this yeast mutant can be cultured by conventional methods as commonly used for culture of yeast. For example, the synthesized medium (containing carbon source, nitrogen source, mineral salts, amino acids, vitamins, etc.) supplemented with various culture-medium ingredients as supplied from Difco and free from amino acids as supplied by a marker required for duplication and maintenance of the plasmid can be used (Scherman, Methods Enzymol., 194, 3-57 (1991)).

The culture medium for expression of a heterologous gene by an expression vector which is controlled by a methanol-inducible promoter to produce the desired gene expression product may contain a compound which has an oxygen atom(s) or a nitrogen atom(s) and at least one C1 substituent which binds to the atom. For example, methanol can be added as the compound which has an oxygen atom, and at least one compound selected from the group consisting of methylamine, dimethylamine, trimethylamine, and an ammonium compound with N-substituted methyl (e.g., choline) can be added as the compound having a nitrogen atom(s).

The medium may contain, in addition to methanol as the carbon source, one or more nitrogen sources such as yeast extract, tryptone, meat extract, casamino acid and ammonium salt, and mineral salts such as phosphate, sodium, potassium, magnesium, calcium, iron, copper, manganese and cobalt, and if necessary, trace nutrients such as various types of vitamins and nucleotide, and appropriately carbohydrate materials for growth of yeast cells before the methanol induction. Specifically, the medium includes YPM medium (0.67% yeast nitrogen base, 1% yeast extract, 2% peptone, 0.5% methanol), BYPM medium (0.67% yeast nitrogen base, 1% yeast extract, 2% peptone, 0.5% methanol, 0.1M phosphate buffer pH 6.0), BM medium (0.67% yeast nitrogen base, 0.5% methanol, 0.1M phosphate buffer pH 6.0), etc.

The culture medium for expressing heterologous genes by an expression vector, which is controlled by a constitutive expression promoter, to produce a desired gene expression product includes culture mediums suitable for cell growth. For example, synthesized media

such as natural culture media such as YPD medium (1% yeast extract, 2% peptone, 2% glucose) and SD medium (0.67% yeast nitrogen base, 2% glucose) can be used. Complementary nutrients may be supplemented in the above-mentioned media for yeast strains having an auxotrophic marker.

pH of the culture medium is suitably adjusted to 5.5 to 6.5. Culture temperature is 15-30°C, preferably around 28°C. When the protein has a complex conformation like an antibody, culturing at low temperature is desirable in order to perform folding more efficiently within the cell. Culture time is about 24-1,000 hours, and culture can be conducted by means of standing culture, shaking culture, stirring culture, batch culture or continuous culture under aeration, or the like.

Conventional methods for isolation and purification of proteins can be used for isolating and purifying the expression product of a heterologous gene from the above-mentioned culture (i.e., culture broth or cultured cells).

For example, the cells may be collected by centrifugation after the culture, suspended in an aqueous buffer, and disrupted by ultrasonicator, French press, Manton-Gaulin homogenizer, Dynomill or the like, to obtain a cell-free extract. When the desired protein is produced in the culture supernatant, the culture broth itself can be used. If necessary, a protease inhibitor may be added to the medium. It is effective to use a protease deficient strain in order to suppress degradation of the expression product of a heterologous gene. Purified preparation or standard can be obtained by a conventional method for isolating and purifying proteins, from the supernatant obtained by centrifugation of the cell-free extract or supernatant. Specifically, the purification can be conducted by using: for example, removal of nucleic acids by protamine treatment; precipitation by fractionating with ammonium sulfate, alcohol, acetone added; anion exchange chromatography using resins such as DEAE Sepharose and Q Sepharose; cation exchange chromatography using resins such as S-Sepharose FF (Pharmacia); hydrophobic chromatography using resins such as butylsepharose and phenylsepharose; gel filtration using molecular sieves; chelate columns such as His Bind resin (Novagen); affinity chromatography using resins such as Protein A Sepharose, specific dye-adsorbed resins such as Blue Sepharose; or lectin columns such as a

ConA Sepharose; reverse phase chromatography; chromatofocusing; and electrofocusing; electrophoresis using polyacrylamide gel, singly or in combination, thereby to obtain the purified preparation or standard. However, the above-mentioned culture and purification methods are specific examples and are not limited thereto.

The amino acid sequence of the purified gene product can be identified by the known amino acid analyses, such as the automated amino acid sequencing using the Edman degradation method.

Examples

The invention will now be described in detail with reference to specific examples. These are for illustrative purposes only, and are not intended to be limiting in any way the scope of the invention. The plasmids, enzymes such as restriction enzymes, T4 DNA ligase, and other substances are all commercially available and can be used by conventional methods. Manipulations used in DNA cloning, sequencing, transformation of host cells, culture of transformed cells, harvest of enzymes from resultant cultures, purification, etc. are also well known to those skilled in the art or can be known from the literature.

The restriction sites in restriction maps of various types of genes are shown by the following abbreviation. Ac; AccI, Ap; ApaI, Bl; BalI, Bm; BamHI, Bg; BglII, Bt;BtgI, Bw; BsiWI, Cl; ClaI, RI; EcoRI, RV; EcoRV, TI; EcoT22I, Hc; HincII, Hd; HindIII, Kp; KpnI, Nd; NdeI, Nh; NheI, Nt; NotI, Pf; PflMI, Pm; PmaCI, Ps; PstI, Sc; SacI, Sl; SalI, Sm; SmaI, Sp; SpeI, Sh; SphI, Su; StuI, St; StyI, Xb; XbaI, and Xh; XhoI.

Example 1

Selection of methylotrophic yeast suitable for production of mammalian type sugar chain

To obtain a mammalian type sugar chain producing yeast using methylotrophic yeast, it is necessary to clone and inactivate a sugar chain synthesizing gene peculiar to the methylotrophic yeast. The sugar chain structure differs largely with the type of the yeast, as described above. In other words, the enzyme and gene involved in the biosynthesis of sugar chain also differ depending on the type of the yeast. Accordingly, when intending to disrupt

the gene involved in the biosynthesis of sugar chain to remove the sugar chain peculiar to the yeast, the first thing to do is to isolate the gene. As such isolation, however, requires a large number of steps, we decided to select a methylotrophic yeast, which requires the smallest possible number of isolation steps. The selection of strains suitable for the isolation was made using NMR data on the cell wall of yeast as an indication of selection (Figure 3) (P.A.J. Gorin et al. (eds), Advanced in Carbohydrate Chemistry and Biochemistry, Vol. 23, 367-417 (1968)). Specifically, in a primary selection, strains suitable for isolation were selected, which had an α-1,2-mannoside linkage-related signal at around 4.3 ppm as a main peak but neither a α-1,3-mannoside linkage-related signal at around 4.4 ppm nor any signals at 4.5 ppm or larger. Then a secondary selection was made by extracting N-linked sugar chains from mannoprotein on the surface of the cells from the yeast strains and analyzing the extracted sugar chains by α-1,2-mannosidase digestion and HPLC. The methylotrophic yeast for the secondary selection were *Candida succiphila* IFO 1911 and *Ogataea minuta* IFO 10746. At the same time, both of *Saccharomyces cerevisiae* having α-1,3-mannoside linkage at unreduced termini of sugar chains, and *Candida boidinii* ATCC 48180 which is a methylotrophic yeast having a peak at 4.5 ppm or larger on the above NMR data, were also analyzed as controls.

Fifty ml of YPD medium containing the above strains was put into a 500 ml Sakaguchi flask, and cultured at 30°C for 24-48 hours, and cells were harvested from the culture by centrifugation, suspended in 10 ml of 100 mM sodium citrate buffer (pH 7.0) and heated in autoclave at 121°C for 1 hour. After cooling, the suspension was centrifuged to collect the supernatant, 10 ml of water was added to the solid matter, and a mixture was heated in the same manner as above and centrifuged to collect the supernatant. The combined cell extracts were poured into 3 volumes of ethanol. The resultant white precipitate was dried, which was then dissolved in concanavalin A (ConA) column buffer (0.1 M sodium phosphate buffer containing 0.15 M sodium chloride, 0.5 mM calcium chloride (pH 7.2)), applied to a ConA-agarose column (0.6 × 2 cm, Honen Corporation), washed with ConA column buffer, and eluted with ConA column buffer containing 0.2 M α-methylmannoside. Concanavalin A is a lectin that has an affinity for sugar chains containing two or more α-D-mannose residues

whose C-3, C-4 and C-6 hydroxyl groups remain unsubstituted, and the column with immobilized lectin enables the separation of mannan protein from glucan, chitin and the like, which are yeast cell wall polysaccharides (Peat et al. J. Chem. Soc., 29 (1961)). The resultant fraction was dialyzed and freeze-dried to yield mannan protein.

Then, the obtained mannan protein was treated with enzyme to cut out Asn-linked sugar chains. Specifically, the freeze-dried standard was dissolved in 100 μl of N-glycosidase F buffer (0.1 M Tris-HCl buffer containing 0.5% SDS, 0.35% 2-mercaptoethanol (pH 8.0)) and boiled for 5 minutes. After cooling the boiled solution to room temperature, 50 μl of 7.5% Nonidet P-40, 138 μl of H_2O and 12 μl of N-glycosidase F (Boehringer Ingelheim) were added and treated at 37°C for 16 hours. After desalting with a BioRad AG501-X8 column, the equal amount of phenol : chloroform (1 : 1) was added and vigorously shaken to remove the detergent and proteins, to yield a sugar chain preparation.

To fluorescence-label (pyridylamination; referred to as PA) the obtained sugar chains, the following were carried out. After concentrating the sugar chain preparation to dryness, 40 μl of a coupling agent (552 mg of 2-aminopyridine dissolved in 200 μl of acetic acid) was added, sealed, and treated at 90°C for 60 minutes. After cooling to room temperature, 140 μl of a reducing agent (200 mg of borane-dimethylamine complex dissolved in 50 μl of H_2O and 80 μl of acetic acid) was added, sealed, followed by treating at 80°C for 80 minutes. After reaction, 200 μl of aqueous ammonia was added, the equal amount of phenol : chloroform (1 : 1) was added and vigorously shaken to recover the water layer that contained PA-oligosaccharides. A series of the steps was repeated 7 times to remove unreacted 2-aminopyridine. The supernatant was filtered through a 0.22 μm filter to yield a PA-oligosaccharide preparation.

The obtained sugar chains were cleaved with *Aspergillus saitoi* α-1,2-mannosidase (SEIKAGAKU CORPORATION, Japan) and then analyzed by HPLC. HPLC using an amide column enables PA-oligosaccharides to be separated depending on the chain length. The HPLC conditions were as follows.

Column: TSK-Gel Amido-80 (4.6 × 250 mm, TOSOH CORPORATION, Japan)

Column temperature: 40°C

Flow rate: 1 ml

Elution conditions: A: 200 mM triethylamine acetate pH 7.0 + 65% acetonitrile

B: 200 mM triethylamine acetate pH 7.0 + 30% acetonitrile

Linear gradient of 0 minute A = 100% and 50 minutes A = 0%

Excitation wavelength: 320 nm

Fluorescence wavelength: 400 nm

The results are shown in Figure 4. The results revealed that N-linked sugar chains derived from *Ogataea minuta* and *Candida Succiphila* were degraded to small molecules of Man5 or Man6 by α-1,2-mannosidase treatment, and thus suggested that sugar chain mutants (Man5 producing strains) corresponding to *och1*, *mnn1* and *mnn4* in *Saccharomyces cerevisiae* could be prepared by inactivation of *OCH1* gene and expression of α-1,2-mannosidase. On the other hand, for *Candida boidinii*, sugar chains remained undegraded at a considerably high rate. This is possibly due to the linkage of a unit other than α-1,2-mannosidic linkage at the terminus of the sugar chains. Similarly, for *Saccharomyces cerevisiae* as the control, there existed sugar chains undegraded, because possible addition of α-1,3-mannose resulting from the action of *MNN1* gene.

Example 2

Cloning of glyceraldehyde-3-phosphate dehydrogenase (GAP) gene of *Ogataea minuta*

The *GAP* gene was obtained from *Ogataea minuta* IFO 10746 and its nucleotide sequence was determined.

(2-1) Preparation of Probe

Oligonucleotides comprising nucleotide sequences corresponding to the following amino acid sequences conserved in glyceraldehyde-3-phosphate dehydrogenases from *Saccharomyces cerevisiae* (GenBank accession number; P00359) and from *Pichia pastoris* (GenBank accession number; Q92263):

AYMFKYDSTHG (SEQ ID NO:1); and

DGPSHKDWRGG (SEQ ID NO:2)

were synthesized as follows.

PGP5; 5'-GCNTAYATGTTYAARTAYGAYWSNACNCAYGG-3' (SEQ ID NO:3)

PGP3; 5'-CCNCCNCKCCARTCYTTRTGNSWNGGNCCRTC-3' (SEQ ID NO:4)

The primer PGP5 has a sequence complementary to the nucleotide sequence corresponding to the amino acid sequence AYMFKYDSTHG, and the primer PGP3 has a sequence complementary to the nucleotide sequence corresponding to the amino acid sequence DGPSHKDWRGG.

Chromosomal DNA was prepared from the cells of *Ogataea minuta* IFO 10746, which were cultured until stationary phase in YPD medium (comprising 1% yeast extract, 2% peptone, 2% glucose, pH 6.0), by means of potassium acetate method (Methods in yeast genetics (1986), Cold Spring Harbor Laboratory, Cols Spring Harbor, New York).

PCR by Ex Taq polymerase (TAKARA SHUZO CO., LTD., Japan) ((94°C for 30 seconds, 50°C for 1 minute and 72°C for 45 seconds) × 25 cycles) was carried out using the obtained chromosomal DNA of *Ogataea minuta* IFO 10746, as a template, and primers PGP5, PGP3. An amplified DNA fragment of approximately 0.5 kb was recovered and cloned using TOPO TA Cloning Kit (Invitrogen). Plasmid DNA was isolated from the obtained clones and sequenced using BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems). For a DNA insert of the plasmid, a clone was selected, which had a nucleotide sequence encoding an amino acid sequence having a high homology with the amino acid sequences for GAP genes from *Saccharomyces cerevisiae* and *Pichia pastoris.* The 0.5-kb DNA insert was recovered after EcoRI digestion of the plasmid and agarose gel electrophoresis.

(2-2) Construction of library and screening

The chromosomal DNA of *Ogataea minuta* IFO 10746 was cleaved with different restriction enzymes and subjected to 0.8% agarose gel electrophoresis. The separated DNA was transferred to Hybond N+ nylon membrane (Amersham). The DNA fragment obtained in Example (2-1) was radiolabeled using Megaprimar DNA Labeling System (Amersham) and subjected to Southern analysis. The hybridization was carried out by conventional procedure (Molecular cloning 2nd edn., ed. Sambrook, J., et al., Cold Spring Harbor Laboratory U.S.A.,

1989). The results suggested that there existed a GAP gene in the HindIII-EcoRV fragment of approximately 6 kb. Then, to clone the DNA fragment, a library was constructed. The chromosomal DNA of *Ogataea minuta* was cleaved with HindIII and EcoRV and subsequently electrophoresed on agarose gel, and the approximately 6-kb DNA fragment was recovered from the gel. The recovered DNA fragment was ligated with HindIII- and HincII-cleaved pUC118 and then transformed into *Escherichia coli* DH5 α strain by the Hanahan method (Gene, *10*, 63 (1980)) to obtain a library.

Approximately 4,000 clones were screened by colony hybridization using the above described DNA fragment as a probe. A clone bearing plasmid pOMGP1 was selected from the 11 positive clones obtained.

(2-3) Sequencing of nucleotide sequence

The nucleotide sequence of the HindIII-BamHI region of the plasmid pOMGP1 (Fig. 5) was determined by deletion mutant and primer walking method using Double-Stranded Nested Deletion Kit (Pharmacia). The nucleotide sequence represented by SEQ ID NO:5 was determined by aligning the obtained nucleotide sequences.

In the nucleotide sequence of SEQ ID NO:5 there existed an open reading frame of 1,011 bp, starting at position 1,492 and ends at position 2,502. The homology studies between the amino acid sequence (SEQ ID NO:6) deduced from the open reading frame and the glyceraldehyde-3-phosphate dehydrogenase from *Saccharomyces cerevisiae* or *Pichia pastoris* showed that 77% or 81% of amino acids were respectively identical between them.

Example 3

Construction of expression cassette using GAP gene promoter and terminator

An expression cassette for transferring foreign genes was constructed between the *GAP* gene promoter (SEQ ID NO:7) and terminator (SEQ ID NO: 8) of *Ogataea minuta*. A 3.2-kb HindIII-BamHI fragment was isolated from pOMGP1 described in Example 2-2 and inserted into the HindIII-BamHI of pBluscript II SK-. The obtained plasmid was named pOMGP2 (Fig. 5). A 3-kb HindIII-KpnI fragment was isolated from the pOMGP2 and the EcoRI site

was inserted into the HindIII-KpnI of blunt-ended pUC19. The resultant plasmid was named pOMGP3 (Fig. 5). To transfer SalI and EcoT22I sites between the *GAP* gene promoter and terminator, the primers:

5'-GTTTGAATTCACTCAATTAACATACACAAATACAATACAAAGTCGACAAAAA ATGCATGTGGATAGATGACCAATGGCCTCTTTAAGTAAACATTTCGTTTTGAATAT ATTTC-3' (SEQ ID NO:9), and

5'-TTTTTACTAGTACGGTACCGCTCGAATCGACACAGGAG-3' (SEQ ID NO:10)

were synthesized. These primers were used to carry out PCR using the pOMGP2 as a template ((94°C for 30 seconds, 55°C for 1 minute and 72°C for 45 seconds) × 20 cycles)). An amplified DNA fragment of approximately 0.6 kb was recovered and cloned using TOPO TA Cloning Kit. An inserted DNA fragment of 0.6 kb was isolated as an EcoRI-KpnI fragment and inserted into the EcoRI-KpnI of the pOMGP3. The obtained plasmid was named pOMGP4 (Figure 5). The pOMGP4 comprises an expression cassette controlled by *GAP* gene promoter and terminator, which cassette allows foreign genes to transfer into SalI-EcoT22I.

Example 4

Construction of G418 resistant gene expression cassette

To perform the transformation comprising selection of an antibiotic G418 resistant gene, a plasmid was constructed which comprised an expression cassette of a G418 resistant gene (aminoglycoside phosphotransferase gene). A 1.1-kb G418 resistant gene isolated, as a XhoI-PstI fragment, from plasmid pUC4K (Amersham Pharmacia) was inserted into the SalI-EcoT22I of the pOMGP4 constructed in Example 3. The resultant plasmid was named pOMKmR1.

Example 5

Cloning of orotidin-5'-phosphate decarboxylase (*URA3*) gene of *Ogataea minuta*

The *URA3* gene was obtained from *Ogataea minuta* IFO 10746, and its nucleotide sequence was determined.

(5-1) Preparation of Probe

Oligonucleotides having the nucleotide sequences corresponding to the amino acid sequences conserved in orotidin-5'-phosphate decarboxylases from *Saccharomyces cerevisiae* (GenBank accession number; K02207) and *Pichia pastoris* (GenBank accession number; AF321098):

GPYICLVKTHID (SEQ ID NO:11); and

GRGLFGKGRDP (SEQ ID NO:12)

were synthesized as follows.

PUR5; 5'-GGNCCNTAYATHTGYYTNGTNAARACNCAYATHGA-3' (SEQ ID NO:13)

PUR3; 5'-GGRTCNCKNCCYTTNCCRAANARNCCNCKNCC-3' (SEQ ID NO:14)

The primer PUR5 has a sequence complementary to the nucleotide sequence corresponding to the amino acid sequence GPYICLVKTHID, and the primer PUR3 has a sequence complementary to the nucleotide sequence corresponding to the amino acid sequence GRGLFGKGRDP.

PCR by primers PUR5 and PUR3 was performed using the chromosomal DNA of *Ogataea minuta* IFO 10746 as a template ((94°C for 30 seconds, 50°C for 1 minute and 72°C for 30 seconds) × 25 cycles). The amplified DNA fragment of approximately 0.6 kb was recovered and cloned using TOPO TA Cloning Kit. Plasmid DNA was isolated from the obtained clone and sequenced. For a DNA insert of the plasmid, a clone was selected which had a nucleotide sequence encoding an amino acid sequence highly homologous to the amino acid sequences of orotidin-5'-phosphate decarboxylases from *Saccharomyces cerevisiae* and *Pichia pastoris*. The 0.6-kb DNA insert was recovered after EcoRI cleavage of the plasmid and agarose gel electrophoresis.

(5-2) Preparation of library and screening

The chromosomal DNA of *Ogataea minuta* IFO 10746 was cleaved with different restriction enzymes, and subjected to Southern analysis using the DNA fragment obtained in (5-1) as a probe by the method described in Example (2-2). The results suggested that there was present *URA3* gene in the HindIII fragment of approximately 4.5 kb. Then, to clone the

DNA fragment, a library was constructed. The chromosomal DNA of *Ogataea minuta* was cleaved with HindIII and electrophoresed on agarose gel, and then the approximately 4.5-kb DNA fragment was recovered from the gel. The resultant DNA fragment was ligated with HindIII-cleaved pUC18 and then transformed into *Escherichia coli* DH5 α strain to obtain a library.

Approximately 6,000 clones were screened by colony hybridization using the above described DNA fragment as a probe. A clone bearing plasmid pOMUR1 was selected from the 3 positive clones obtained.

(5-3) Sequencing of nucleotide sequence

The nucleotide sequence of the NotI-HindIII region of the plasmid pOMUR1 (Fig. 6) was determined by deletion mutant and primer walking method to obtain a nucleotide sequence represented by SEQ ID NO:15.

In the nucleotide sequence of SEQ ID NO:15, there existed an open reading frame of 798 bp, starting at position 1,732 and ends at position 2,529. The homology studies between the amino acid sequence (SEQ ID NO:16) deduced from the open reading frame and the orotidin-5'-phosphate decarboxylase from *Saccharomyces cerevisiae* or *Pichia pastoris* showed that 82% or 75% of amino acids were respectively identical between them.

Example 6

Preparation of *Ogataea minuta URA3* knockout mutant

An *Ogataea minuta URA3* knockout mutant was prepared by the "pop-in, pop-out" method (Rothstein R., Methods Enzymol., *194* (1991)).

(6-1) Preparation of *URA3* gene disruption vector

A 3-kb NotI-KpnI fragment was isolated from the plasmid pOMUR1 (Fig. 6) described in Example (5-2) and inserted into the NotI-KpnI of pBluescript II SK-. After cleaving the plasmid with NotI and StyI, plasmid pOMUM1 (Fig. 6) was obtained by blunt-end treatment and self-ligation. Primers 5'-ATGGAGAAAAAAACTAGTGGATATACCACC-3' (SEQ ID NO:17) and 5'-CTGAGACGAAAAAGATATCTCAATAAACCC-3' (SEQ ID NO:18) were

used to carry out PCR using plasmid pHSG398 (TAKARA SHUZO CO., LTD., Japan) as a template ((94°C for 30 seconds, 55°C for 1 minute and 72°C for 45 seconds) × 20 cycles)) to amplify part of chloramphenicol resistant gene. The 0.4-kb amplified DNA fragment was cleaved with SpeI and EcoRV and inserted into the SpeI-RcoRV of the pOMUM1. The obtained plasmid was named pOMUM2.

The plasmid pOMKmR1, which contained the G418 resistant gene expression cassette controlled by the *GAP* gene promoter and terminator as prepared in Example 4, was cleaved with HindIII, blunt-ended, and ligated with a KpnI linker. The G418 resistant gene expression cassette was isolated as a 3-kb KpnI fragment from the plasmid and transferred at KpnI of the pOMUM2. The obtained plasmid was named pDOMU1 (Fig. 6).

(6-2) Transformation

The pDOMU1 constructed in Example (6-1) was cleaved with SalI and transformed into *Ogataea minuta* IFO 10746 by the electric pulse method. The transformants were precultured in YPD medium at 30°C overnight, inoculated into 100 ml of YPD medium, and cultured at 30°C for 8-16 hours until logarithmic growth phase (OD_{600} = about 1.5). The cells were harvested by centrifugation at 1400 × g for 5 minutes, washed once with 100 ml of sterilized ice-cooled water, then once with 40 ml of sterilized ice-cooled water. Then the cells were suspended in 20 ml of LC buffer (100 mM LiCl, 50 mM potassium phosphate buffer, pH 7.5) and shaken at 30°C for 45 minutes, and then 0.5 ml of 1 M DTT was added to the suspension and shaken for another 15 minutes. After washed with 80 ml of ice-cooled STM buffer (270 mM sucrose, 10 mM Tris-HCl buffer, pH 7.5, 1 mM $MgCl_2$), the cells were suspended in 320 μl of STM buffer. The transformation by the electric pulse method was performed with Gene Pulser (BIO-RAD). After mixing 50 μl of the cell suspension and 5 μl of DNA sample, the mixture was put into a 0.2 cm disposable cuvette, and an electric pulse was applied to the mixture under appropriate conditions (voltage: 1.0 to 1.5 kv, resistance: 200-800 Ω). After application of the pulse, 1 ml of ice-cooled YDP medium containing 1 M sorbitol was added and subjected to shaking culture at 30°C for 4-6 hours. After the culture,

the cell liquid was applied on a YPD selection medium containing 400-1000 μg/ml G418, and the plate was incubated at 30°C to obtain transformant colonies.

To confirm that the *URA3* gene was disrupted, the following primers were synthesized (see Fig. 7 with regard to the position of each primer).

DU5; 5'-AGGAAGAAGAGGAGGAAGAGGAAGAAAC-3' (SEQ ID NO:19)

DUC5; 5'-CGATGCCATTGGGATATATCAACGGTGG-3' (SEQ ID NO:20)

DU3; 5'-CCGTGTTTGAGTTTGTGAAAAACCAGGGC-3' (SEQ ID NO:21)

DUC3; 5'-TGTGGCGTGTTACGGTGAAAACCTGGCC-3' (SEQ ID NO:22)

PCR by primers DU5 and DUC5 was performed using the chromosomal DNA isolated from the transformant as a template ((94°C for 30 seconds, 60°C for 1 minute and 72°C for 1 minute) × 25 cycles). As shown in Fig. 7, a 1.1-kb amplified DNA fragment was detected from the strain whose *URA3* locus had the plasmid integrated there into. After culturing the selected strain in the YPD medium until stationary phase, a strain resistant to 5-fluoroorotic acid (5-FOA) was obtained in accordance with the method described in a manual for experimental procedures (Methods Enzymol., 154, 164 (1987)). PCR by primers DU5 and DU3 ((94°C for 30 seconds, 60°C for 1 minute and 72°C for 3 minutes) × 25 cycles), PCR by primers DU5 and DUC5 ((94°C for 30 seconds, 60°C for 1 minute and 72°C for 1 minute) × 25 cycles), and PCR by primers DU3 and DUC3 ((94°C for 30 seconds, 60°C for 1 minute and 72°C for 1 minute) × 25 cycles), were performed using the chromosomal DNA isolated from the 5-FOA resistant strain as a template. As shown in Fig. 7, in the strain in which G418 resistant gene was deleted and the ORF of *URA3* gene was replaced with the chloramphenicol resistant gene region, a 2.6-kb amplified DNA fragment was detected by PCR using DU5 and DU3, a 1.1-kb amplified DNA fragment by PCR using DU5 and DUC5, and a 1.0-kb amplified DNA fragment by PCR using DU3 and DUC3, respectively. The yeast was named *Ogataea minuta* strain TK1-3 (ura3Δ).

Example 7

Cloning of *ADE1* (phosphoribosyl-amino-imidazole succinocarboxamide synthase) gene from *Ogataea minuta*

The *ADE1* gene was obtained from *Ogataea minuta* IFO 10746 and its nucleotide sequence was determined.

(7-1) Preparation of Probe

Oligonucleotides having nucleotide sequences corresponding to the amino acid sequences conserved in the *ADE1* gene products from *Saccharomyces cerevisiae* (GenBank accession number; M61209) and *Candida maltosa* (GenBank accession number; M58322):

FVATDRISAYDVIM (SEQ ID NO:23); and

QDSYDKQFLRDWLT (SEQ ID NO:24)

were synthesized as follows.

PADS5; 5'-TTYGTNGCNACNGAYMGNATHWSNGCNTAYGAYGTNATHATG-3' (SEQ ID NO:25)

PAD3; 5'-GTNARCCARTCNCKNARRAAYTGYTTRTCRTANSWRTCYTG-3' (SEQ ID NO:26)

The primer PAD5 has a sequence complementary to the nucleotide sequence corresponding to the amino acid sequence FVATDRISAYDVIM, and the primer PAD3 has a sequence complementary to the nucleotide sequence corresponding to the amino acid sequence QDSYDKQFLRDWLT.

PCR by primers PAD5 and PAD3 was performed using the chromosomal DNA of *Ogataea minuta* IFO 10746 as a template ((94°C for 30 seconds, 50°C for 1 minute and 72°C for 1 minute) × 25 cycles). The amplified DNA fragment of approximately 0.7 kb was recovered and cloned using TOPO TA Cloning Kit. Plasmid DNA was isolated from the obtained clone and sequenced. For a DNA insert of the plasmid, a clone was selected which had a nucleotide sequence encoding an amino acid sequence highly homologous to the amino acid sequences of the *ADE1* genes from *Saccharomyces cerevisiae* and *Candida maltosa.*

The 0.7-kb DNA insert was recovered after EcoRI cleavage of the plasmid and agarose gel electrophoresis.

(7-2) Preparation of library and screening

The chromosomal DNA of *Ogataea minuta* IFO 10746 was cleaved with different restriction enzymes, and subjected to Southern analysis using the DNA fragment obtained in (7-1) as a probe by the method described in Example (2-2). The results suggested that there existed *ADE1* gene in the approximately 5 kb HindIII-BamHI fragment. Then, to clone the DNA fragment, a library was prepared. The chromosomal DNA of *Ogataea minuta* was cleaved with HindIII and BamHI and electrophoresed on agarose gel, and then the approximately 5-kb DNA fragment was recovered from the gel. The DNA fragment was ligated with HindIII- and BamHI-cleaved pBluescript II SK- and then transformed into *Escherichia coli* strain DH5 α to prepare a library.

Approximately 6,000 clones were screened by colony hybridization using the above described DNA fragment as a probe. A clone bearing plasmid pOMAD1 was selected from the 9 positive clones obtained.

(7-3) Sequencing of nucleotide sequence

The nucleotide sequence of the EcoRV-SmaI region of the plasmid pOMAD1 (Fig. 8) was determined by deletion mutant and primer walking method to obtain a nucleotide sequence represented by SEQ ID NO:27.

In the nucleotide sequence of SEQ ID NO:27, there existed an open reading frame of 912 bp, starting at position 939 and ends at position 1,850. The homology studies between the amino acid sequence (SEQ ID NO:28) deduced from the open reading frame and the *ADE1* gene product from *Saccharomyces cerevisiae* or *Pichia pastoris* showed that 69% or 74% of amino acids were respectively identical between them.

Example 8

Preparation of *Ogataea minuta ADE1* knockout mutant

The *ADE1* gene was disrupted by transformation using the *URA3* gene of *Ogataea minuta* as a marker.

(8-1) Preparation of *ADE1* Disruption Vector

As shown in Fig. 8, plasmid pDOMAD1 was prepared by replacing approximately 70-bp region of the *ADEI* structural gene by the *URA3* gene. To obtain a uracil auxotrophic mutant again from *ADEI* gene knockout mutants, the *URA3* gene having repetitive structures before and after the structural gene was used as a marker. PCR by the primers:

5'-CCCCGAGCTCAAAAAAAAGGTACCAATTTCAGCTCCGACGCCGGAGCCCACT ACGCCTAC-3' (SEQ ID No. 29); and

5'-GGGAAGCTTCCCCAGTTGTACACCAATCTTGTCGACAG-3' (SEQ ID No. 30)

was performed using, as a template, the plasmid pOMUR1 having the *URA3* gene region as described in Example 5 ((94°C for 30 seconds, 50°C for 1 minute and 72°C for 45 seconds) × 20 cycles) to amplify the upstream region of the *URA3* structural gene. The amplified DNA fragment of approximately 0.8 kb was recovered, cleaved with SacI and HindIII, and inserted into the SacI-HindIII of the pUC18.

The 3.3-kb SacI-KpnI fragment isolated from the pOMUR1 was inserted into the SacI-KpnI of the obtained plasmid. The resultant plasmid was cleaved with KpnI, blunt-ended, and self-ligated. The obtained plasmid was named pOMUR2 (Fig. 9). The pOMUR2 was cleaved with StyI, blunt-ended, and ligated with a BglII linker. The obtained plasmid was named pROMU1. In the 3.3-kb DNA fragment obtained by cleaving the pROMU1with BglII and HindIII, there existed approximately 0.8-kb repetitive sequences before and after the *URA3* structural gene (Fig. 9).

PCR by the primers:

Dad1-5:5'-AAAAAGCGGCCGCTCCCGGTGTCCCGCAGAAATCTTTATGCGTAGTCTT G-3' (SEQ ID NO:31); and

Dadl-3:5'-CCCCCGGATCCTTTTTTTTAAGCTTGTTGTACTCCTTCCATGCACTTCCGG TGATG-3' (SEQ ID NO:32)

((94°C for 30 seconds, 50°C for 1 minute and 72°C for 1 minute) × 20 cycles), and

PCR by the primers:

Dad2-5:5'-TTTTCACCCCGTCAAGGATCCCTGAACAAGGCGAACACGACGAAAACA TTTCCCCCGAG-3' (SEQ ID NO:33); and

Dad2-3:5'-TTTTTGGGCCCACCTGGGTGAAGATTTGCCAGATCAAGTTCTCC-3' (SEQ ID NO:34)

((94°C for 30 seconds, 50°C for 1 minute and 72°C for 1 minute) × 20 cycles)

were performed using, as a template, the plasmid pOMAD1 having the *ADE1* gene region as described in Example 7. The amplified DNA fragments of approximately 0.7 kb and 1 kb were recovered and cleaved with NotII and BamHI and with BamHI and ApaI, respectively. Both of the NotI-BamHI and BamHI-ApaI DNA fragments obtained were inserted into the NotI-ApaI of the pBluescript II SK-. The 3.3-kb BglII-HindIII fragment isolated from the pROMU1 was inserted into the BamHI-HindIII of the obtained plasmid. The resultant plasmid was named pDOMAD1 (Fig. 8).

(8-2) Transformation

The pDOMAD1 obtained in Example (8-1) was cleaved with ApaI and NotI and transformed into *Ogataea minuta* strain TK1-3 (ura3Δ) obtained in Example (6-2) by the electric pulse method. Strains exhibiting *ade1* trait produce a red pigment, which is an intermediate metabolite in the adenine biosynthesis, and their colonies are dyed red. Thus, strains whose colonies were dyed red compared with the transformants were selected. To confirm that the *ADE1* genes of these strains were disrupted, the following primers were synthesized (see Fig. 10 with regard to the position of each primer).

DA5; 5'-GATGCTTGCGCCTTCAACCACATACTCCTC-3' (SEQ ID NO:35)

DA3; 5'-AAAAGTTCTTGCACAGCCTCAATATTGACC-3' (SEQ ID NO:36)

DOU5; 5'-ATCGATTTCGAGTGTTTGTCCAGGTCCGGG-3' (SEQ ID NO:37)

PCR by primers DA5 and DOU5 was performed using the chromosomal DNA isolated from the transformant as a template ((94°C for 30 seconds, 60°C for 1 minute and 72°C for 2 minutes) × 25 cycles). As shown in Fig. 10, a 1.6-kb amplified DNA fragment was detected from the strain whose *ADE1* locus had the plasmid integrated thereinto. After culturing the selected strain in the YPD medium until stationary phase, a strain resistant to 5-fluoroorotic acid (5-FOA) was obtained. PCR by primers DA5 and DA3 was performed using the chromosomal DNA isolated from the 5-FOA resistant strain as a template ((94°C for 30

seconds, 60°C for 1 minute and 72°C for 3 minutes) × 25 cycles). As shown in Fig. 10, in the strain in which *URA3* gene was deleted, a 2.9-kb amplified DNA fragment was detected. The ura3Δ ade1Δ strain was named *Ogataea minuta* strain TK4-1.

Example 9

Cloning of *OCH1* gene from *Ogataea minuta*

The *OCH1* gene was obtained from *Ogataea minuta* IFO 10746 and its nucleotide sequence was determined.

(9-1) Preparation of Probe

Oligonucleotides having nucleotide sequences corresponding to the amino acid sequences conserved in *OCH1* gene products from *Saccharomyces cerevisiae* (GenBank accession number; P31755) and *Pichia pastoris* (Japanese Patent Publication (Kokai) No. 9-3097A):

PQH(R)I(V)WQTWKV (SEQ ID NO:38); and

WYARRIQFCQW (SEQ ID NO:39)

were synthesized as follows.

POH5; 5'-CCNCARCRYRTHTGGCARACNTGGAARGT-3' (SEQ ID NO:40)

POH3; 5'-CCAYTGRCARAAYTGDATNCKNCKNGCRTACCA-3' (SEQ ID NO:41)

The primer POH5 has a sequence complementary to the nucleotide sequence corresponding to the amino acid sequence PQH(R)I(V)WQTWKV, and the primer POH3 has a sequence complementary to the nucleotide sequence corresponding to the amino acid sequence WYARRIQFCQW.

PCR by primers POH5 and POH3 was performed using the chromosomal DNA of *Ogataea minuta* IFO 10746 as a template ((94°C for 30 seconds, 50°C for 1 minute and 72°C for 30 seconds) × 25 cycles). The amplified DNA fragment of approximately 0.4 kb was recovered and cloned using TOPO TA Cloning Kit. Plasmid DNA was isolated from the obtained clone and sequenced. For a DNA insert of the plasmid, a clone was selected which had a nucleotide sequence encoding an amino acid sequence highly homologous to the amino acid sequences of *OCH1* gene products from *Saccharomyces cerevisiae* and *Pichia pastoris*.

The 0.4-kb DNA insert was recovered after EcoRI cleavage of the plasmid and agarose gel electrophoresis.

(9-2) Preparation of library and screening

The chromosomal DNA of *Ogataea minuta* IFO 10746 was cleaved with different restriction enzymes, and subjected to Southern analysis using the DNA fragment obtained in Example (9-1) as a probe by the method described in Example (2-2). The results suggested that there existed *OCH1* gene in the XbaI fragment of approximately 5 kb. Then, to clone the DNA fragment, a library was prepared. The chromosomal DNA of *Ogataea minuta* was cleaved with XbaI and subjected to agarose gel electrophoresis, and then the approximately 5-kb DNA fragment was recovered from the gel. The recovered DNA fragment was ligated with XbaI-cleaved pBluescript II SK- and then transformed into *Escherichia coli* DH5 α strains to prepare a library.

Approximately 6,000 clones were screened by colony hybridization using the above described DNA fragment as a probe. A clone bearing plasmid pOMOC1 was selected from the 4 positive clones obtained.

(9-3) Sequencing of nucleotide sequence

The nucleotide sequence of the Bg1II-SpeI region of the plasmid pOMOC1 (Fig. 11) was determined by deletion mutant and primer walking method to obtain a nucleotide sequence represented by SEQ ID NO:42.

In the nucleotide sequence of SEQ ID NO:42 there existed an open reading frame consisting of 1,305 bp, starting at position 508 and ends at position 1,812. The homology studies between the amino acid sequence (SEQ ID NO:43) deduced from the open reading frame and the mannosyltransferase *OCH1* gene product from *Saccharomyces cerevisiae* or *Pichia pastoris* showed that 42% or 29% of amino acids were respectively identical between them. It remains unknown whether or not the *Pichia pastoris*-derived *OCH1* gene disclosed in Japanese Patent Publication (Kokai) No. 9-3097A substantially encodes the *OCH1* (α-1,6 mannosyltransferase), or whether or not the same *Pichia pastoris*-derived *OCH1* gene has the

functions of the *OCH1* gene of *Ogataea minuta* described in this Example and Examples 10 and 11. The reasons are that the homology to the *Pichia pastoris*-derived *OCH1* was 29% in amino acid, and that it has not been studied whether the *Pichia pastoris*-derived *OCH1* has the activity of the *Saccharomyces cerevisiae*-derived *OCH1* (α-1,6 mannosyltransferase).

Example 10

Preparation of *Ogataea minuta*-derived *OCH1* knockout mutant

The *OCH1* gene was disrupted by transformation using the *URA3* gene of *Ogataea minuta* as a marker.

(10-1) Preparation of *OCH1* gene disruption vector

Plasmid pDOMOCH1 was prepared by replacing approximately 0.5-kb BalI-SmaI region of the *OCH1* gene by the *URA3* gene (Fig. 11). To obtain a uracil auxotrophic mutant again from *OCH1* knockout mutant, the *URA3* gene having repetitive structures before and after the structural gene, as described in Example (8-1), was used as a marker.

The 4.4-kb NotI-XbaI fragment was isolated from the pOMOC1 and inserted into the NotI-XbaI of pBluescript II SK-. The obtained plasmid was named pOMOC2. The pOMOC2 was cleaved with AccI and XhoI, blunt-ended, and self-ligated. The obtained plasmid was named pOMOC3. The pOMOC2 was cleaved with BalI, and ligated with a BamHI linker. The obtained plasmid was named pOMOC2B (Figure 11). The pOMOC3 was cleaved with SmaI, and ligated with a HindIII linker. The obtained plasmid was named pOMOC3H (Fig. 11). The 3.3-kb BglII-HindIII fragment isolated from the pROMU1 described in Example (8-1) was inserted into the BamHI-HindIII of the pOMOC2B. The 1.5-kb HindIII-ApaI fragment isolated from the pOMOC3H was inserted into the HindIII-ApaI of the obtained plasmid. The resultant plasmid was named pDOMOCH1.

(10-2) Transformation

The pDOMOCH1 obtained in Example (10-1) was cleaved with ApaI and NotI, and transformed into *Ogataea minuta* TK1-3 strain (ura3Δ), which was obtained in Example (6-2), and into *Ogataea minuta* TK4-1 strain (ura3Δ Adel1Δ), which was obtained in Example (8-2),

by electric pulse method. The transformation was performed in accordance with the method described in Example (6-2).

To confirm that the *OCH1* genes of these strains were disrupted, the following primers were synthesized (see Fig. 12 with regard to the position of each primer).

DO3; 5'-CCATTGTCAGCTCCAATTCTTTGATAAACG-3' (SEQ ID NO:44)

DOU5; 5'-ATCGATTTCGAGTGTTTGTCCAGGTCCGGG-3' (SEQ ID NO:37)

DO5; 5'-ACACTTCCGTAAGTTCCAAGAGACATGGCC-3' (SEQ ID NO:45)

DO3-2; 5'-TCACCACGTTATTGAGATAATCAAACAGGG-3' (SEQ ID NO:46)

PCR by primers DO5 and DOU5 was performed using the chromosomal DNA isolated from the transformant as a template ((94°C for 30 seconds, 60°C for 1 minute and 72°C for 3 minutes) × 25 cycles). As shown in Fig. 12, a 2.4-kb amplified DNA fragment was detected in the strain whose *OCH1* locus had the plasmid integrated thereinto. After culturing the selected strain in the YPD medium until stationary phase, a strain resistant to 5-fluoroorotic acid (5-FOA) was obtained. PCR by primers DO3 and DO5 ((94°C for 30 seconds, 60°C for 1 minute and 72°C for 3 minutes) × 25 cycles) and PCR by primers DO5 and DOC3-2 ((94°C for 30 seconds, 60°C for 1 minute and 72°C for 1 minute) × 25 cycles) were performed using the chromosomal DNA isolated from the 5-FOA resistant strain as a template. As shown in Fig. 12, in the strain in which *URA3* gene was deleted, a 2.4-kb amplified DNA fragment was detected by the PCR using primers DO3 and DO5 and a 0.9 kb amplified DNA fragment by the PCR using primers DO5 and DOC3-2. The och1Δ ura3Δ strain obtained was named *Ogataea minuta* TK3-A strain, and the och1Δ ura3Δ ade1Δ strain was named *Ogataea minuta* TK5-3 strain.

Example 11

Isolation of cell surface mannan protein from *Ogataea minuta OCH1* knockout mutant and structure analysis of sugar chain contained therein

Structure analysis of sugar chains of cell surface mannan proteins was performed for *Ogataea minuta OCH1* knockout mutant strainTK3-A and its parent strain TK1-3. The preparation of PA-oligosaccharides was performed by the method described in Example 1.

The prepared sugar chains were cleaved with *Aspergillus saitoi* α-1,2-mannosidase (SEIKAGAKU CORPORATION, Japan). Analysis was performed by HPLC. HPLC on amide column enables PA-oligosaccharides to be separated depending on the chain length. HPLC using a reverse-phase column enables PA-oligosaccharides to be separated depending on the hydropholicity, thereby to identify sugar chain structures. The HPLC conditions were as follows.

1) Size analysis by amide column

Column: TSK-Gel Amido-80 (4.6 × 250 mm, TOSOH CORPORATION, Japan)

Column temperature: 40°C

Flow rate: 1 ml

Elution conditions: A: 200 mM triethylamine acetate pH 7.0 + 65% acetonitrile

B: 200 mM triethylamine acetate pH 7.0 + 30% acetonitrile

Linear gradient of 0 minute A = 100% and 50 minutes A = 0%

2) Structure analysis by reverse phase column

Column: TSK-Gel ODS80TM (4.6 × 250 mm, TOSOH CORPORATION, Japan)

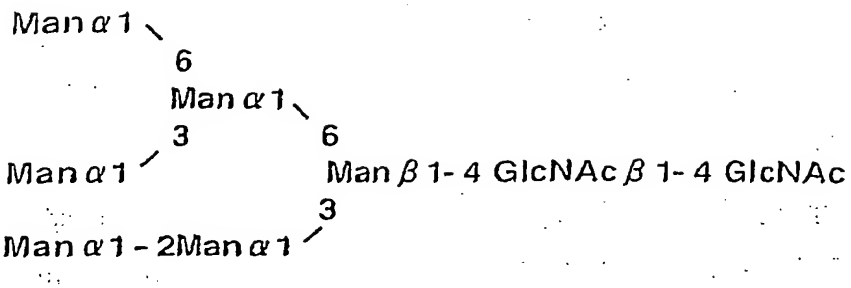
Column temperature: 50°C

Flow rate: 1.2 ml

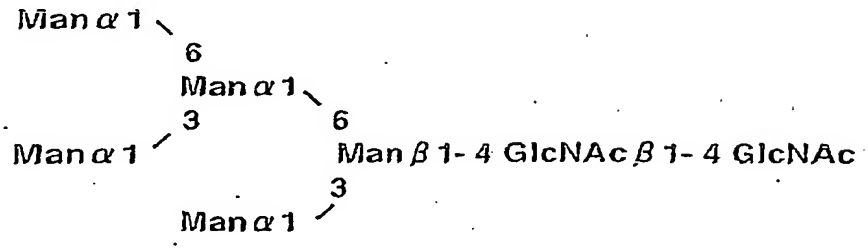
Elution conditions: 100 mM ammonium acetate containing 0.15% n-butanol pH 6.0

The results are shown in Fig. 13. From the size analysis using an amide column, it was confirmed that the TK1-3 strain as a parent strain produced both Man5 and Man6 as shown in Fig. 13, whereas the TK3-A strain, i.e., a *ΔOCH1* strain, mainly produced Man5. Further, from the structure analysis using a reverse phase column and the comparison with commercially available standard sugar chains (TAKARA SHUZO CO., LTD., Japan), it was found that Man6 of the TK1-3 strain was a sugar chain having the structural formula 1 below, Man5 of the TK1-3 strain a sugar chain having the structural formula 2 below, and Man5 of the TK3-A strain a sugar chain having the structural formula 2 below.

Structural Formula 1



Structural Formula 2



From the above results, it was confirmed that the obtained gene was substantially *Ogataea minuta OCH1* gene and that it was possible to prepare sugar chain mutants corresponding to the *och1*, *mnn1* and *mnn4* strains in *Saccharomyces cerevisiae* in which α-1,2-mannosidase gene was expressed.

Example 12

Cloning of proteinase A (*PEP4*) gene of *Ogataea minuta*

The *PEP4* gene was obtained from *Ogataea minuta* IFO 10746 and its nucleotide sequence was determined.

(12-1) Preparation of probe

Oligonucleotides having nucleotide sequences corresponding to the following amino acid sequences conserved in *PEP4* gene from *Saccharomyces cerevisiae* (GenBank accession number; M13358) and *Pichia angusta* (GenBank accession number; U67173):

TNYLNAQY (SEQ ID NO:47); and

KAYWEVKF (SEQ ID NO:48)

were synthesized as follows.

PPA5; 5'-ACNAAYTAYYTNAAYGCNCARTA-3' (SEQ ID NO:49)

PPA3; 5'-AAYTTNACYTCCCARTANGCYTT-3' (SEQ ID NO:50)

The primer PPA5 has a sequence complementary to the nucleotide sequence corresponding to the amino acid sequence TNYLNAQY, and the primer PPA3 has a sequence complementary to the nucleotide sequence corresponding to the amino acid sequence KAYWEVKF.

PCR by primers PPA5 and PPA3 was performed using the chromosomal DNA of *Ogataea minuta* IFO 10746 as a template ((94°C for 30 seconds, 55°C for 1 minute and 72°C for 1 minute) × 25 cycles). The amplified DNA fragment of approximately 0.6 kb was recovered and cloned using TOPO TA Cloning Kit. Plasmid DNA was isolated from the obtained clone and sequenced. For a DNA insert of the plasmid, a clone was selected which had a nucleotide sequence encoding an amino acid sequence highly homologous to the amino acid sequences for *PEP4* genes from *Saccharomyces cerevisiae* and *Pichia angusta.* The 0.6-kb DNA insert was recovered after EcoRI cleavage of the plasmid and agarose gel electrophoresis.

(12-2) Preparation of library and screening

The chromosomal DNA of *Ogataea minuta* IFO 10746 was cleaved with different restriction enzymes, and subjected to Southern analysis using the DNA fragment obtained in Example (12-1) as a probe by the method described in Example (6-2). The results suggested that there existed *PEP4* gene in the approximately 6 kb BamHI fragment. Then, to clone the DNA fragment, a library was prepared. The chromosomal DNA of *Ogataea minuta* was cleaved with BamHI and subjected to agarose gel electrophoresis, and then the approximately 6-kb DNA fragment was recovered from the gel. The recovered DNA fragment was ligated with BamHI-cleaved pUC18 and then transformed into *Escherichia coli* strain DH5 α to prepare a library.

About 5,000 clones were screened by colony hybridization using the above described DNA fragment as a probe. A clone bearing plasmid pOMPA1 was selected from the 8 positive clones obtained.

(12-3) Sequencing of nucleotide sequence

The nucleotide sequence of the NdeI-XbaI region of the plasmid pOMPA1 (Fig. 14) was determined by deletion mutant and primer walking method to obtain a nucleotide sequence represented by SEQ ID NO:51.

In the nucleotide sequence represented by SEQ ID NO:51, there existed an open reading frame of 1,233 bp, starting at position 477 and ends at position 1,709. The homology studies between the amino acid sequence (SEQ ID NO:52) deduced from the open reading frame and the *PEP4* from *Saccharomyces cerevisiae* or *Pichia angusta* showed that 67% or 78% of amino acids were respectively identical between them.

Example 13

Preparation of *Ogataea minuta PEP4* knockout mutant

The *PEP4* gene was disrupted by transformation using the *URA3* gene of *Ogataea minuta* as a marker.

(13-1) Preparation of *PEP4* Disruption Vector

As shown in Fig. 14, plasmid pDOMPA1 was prepared by replacing the approximately 1.1-kb SmaI-XbaI region of the *PEP4* structural gene by the *URA3* gene. To obtain a uracil auxotrophic mutant again from *PEP4* knockout mutants, the *URA3* gene having repetitive structures before and after the structural gene was used as a marker. Plasmid was prepared by SacI cleavage, self-ligation, ClaI cleavage, and self-ligation of the plasmid pOMPA1 carrying the *PEP4* gene region, as described in Example (12-2).

The obtained plasmid was cleaved with SmaI, ligated with a HindIII linker, cleaved with XbaI, blunt0ended, and ligated with a BglII linker.

The 3.3-kb BglII-HindIII fragment isolated from the pROMUI described in Example (8-1) was inserted into the BglII-HindIII of the obtained plasmid. The resultant plasmid was named pDOMPA1 (Fig. 14).

(13-2) Transformation

The pDOMPA1 obtained in Example (13-1) was cleaved at SacI-ClaI, and then transformed into the *Ogataea minuta* TK3-A strain (och1Δ ura3Δ) and the *Ogataea minuta* TK5-3 strain (och1Δ ura3Δ ade1Δ) obtained in Example (10-2), by means of the electric pulse method.

The *PEP4* knockout mutants were screened by subjecting the chromosomal DNAs of the obtained transformants to Southern analysis. Specifically, when cleaving the chromosomal DNAs of the host strain and the transformants with BamHI and subjecting the cleaved chromosomal DNAs to Southern analysis using the 4.8-kb SacI-ClaI fragment isolated from the pDOMPA1 (Fig. 14) as a probe, a band was detected at 6 kb in the host strain, while a band was detected at 9 kb in the knockout mutants. After culturing the knockout mutants in the YPD medium until stationary phase, a strain resistant to 5-fluoroorotic acid (5-FOA) was obtained. The chromosomal DNA of the 5-FOA resistant strain was cleaved with BamHI and again subjected to Southern analysis using the 4.8-kb SacI-ClaI fragment isolated from the pDOMPA1 (Fig. 14) as a probe, and a strain was selected from which the *URA3* gene was deleted and in which a band was detected at 5.5 kb. The och1Δ pep4Δ ura3Δ strain obtained was named *Ogataea minuta* TK6 strain, and the och1Δ pep4Δ ura3Δ ade1Δ strain was named *Ogataea minuta* TK7 strain.

Example 14

Cloning of *PRB1* gene of *Ogataea minuta*

The *PRB1* gene was obtained from *Ogataea minuta* IFO 10746 and its nucleotide sequence was determined.

(14-1) Preparation of Probe

Oligonucleotides having nucleotide sequences corresponding to the following amino acid sequences conserved in *PRB1* from *Saccharomyces cerevisiae* (GenBank accession number; M18097) and *Kluyveromyces lactis* (GenBank accession number; A75534) and their homologues:

DG(L)NGHGTHCAG (SEQ ID NO:53)

GTSMAS (T) PHV (I) A (V) G (SEQ ID NO:54)

were synthesized as follows.

PPB5; 5'-GAYBKNAAYGGNCAYGGNACNCAYTGYKCNGG-3' (SEQ ID NO:55)

PPB3; 5'-CCNRCNAYRTGNGGNWSNGCCATNWSNGTNCC-3' (SEQ ID NO:56)

The primer PPB5 has a sequence complementary to the nucleotide sequence corresponding to the amino acid sequence DG(L)NGHGTHCAG, and the primer PPB3 has a sequence complementary to the nucleotide sequence corresponding to the amino acid sequence GTSMAS(T)PHV(I)A(V)G.

PCR by primers PPB5 and PPB3 was performed using the chromosomal DNA of *Ogataea minuta* IFO 10746 as a template ((94°C for 30 seconds, 50°C for 1 minute and 72°C for 1 minute) × 25 cycles). The amplified DNA fragment of approximately 0.5 kb was recovered and cloned using TOPO TA Cloning Kit. Plasmid DNA was isolated from the obtained clone and sequenced. For a DNA insert of the plasmid, a clone was selected which had a nucleotide sequence encoding an amino acid sequence highly homologous to the amino acid sequences for *PRB1* genes from *Pichia pastoris* and *Kluyveromyces lactis.* The 0.5-kb DNA insert was recovered after EcoRI cleavage of the plasmid and agarose gel electrophoresis.

(14-2) Preparation of library and screening

The chromosomal DNA of *Ogataea minuta* IFO 10746 was cleaved with different restriction enzymes, and subjected to Southern analysis using the DNA fragment obtained in Example (14-1) as a probe by the method described in Example (2-2). The results suggested that there existed *PRB1* gene in the BamHI fragment of approximately 5 kb. Then, to clone the DNA fragment, a library was prepared. The chromosomal DNA of *Ogataea minuta* was

cleaved with BamHI and electrophoresed on agarose gel, and then the approximately 5-kb DNA fragment was recovered from the gel. The DNA fragment was ligated with BamHI-cleaved and BAP-treated pUC18 and then transformed into *Escherichia coli* strain DH5 α to prepare a library.

About 6,000 clones were screened by colony hybridization using the above described DNA fragment as a probe. A clone bearing plasmid pOMPB1 was selected from the 2 positive clones obtained.

(14-3) Sequencing of nucleotide sequence

The nucleotide sequence of the BamHI-HindIII region of the plasmid pOMPB1 (Fig. 15) was determined by deletion mutant and primer walking method to obtain a nucleotide sequence represented by SEQ ID NO:57.

In the nucleotide sequence of SEQ ID NO:57, there existed an open reading frame of 1,620 bp, starting at position 394 and ends at position 2,013. The homology studies between the amino acid sequence (SEQ ID NO:) deduced from the open reading frame and the *PRB1* gene product from *Pichia pastoris* or *Kluyveromyces lactis* showed that 47% or 55% of amino acids were respectively identical between them.

Example 15

Preparation of *Ogataea minuta* PRB1 knockout mutant

The *PRB1* gene was disrupted by transformation using the *URA3* gene of *Ogataea minuta* as a marker.

(15-1) Preparation of *PRB1* gene disruption vector

As shown in Fig. 15, plasmid pDOMPB1 was prepared by replacing the approximately 0.2-kb ClaI-SphI region of the *PRB1* structural gene by the *URA3* gene. To obtain a uracil auxotrophic mutant again from *PRB1* knockout mutants, the *URA3* gene having repetitive structures before and after the structural gene was used as a marker. The BamHI fragment was isolated from the plasmid pOMPB1 having the *PRB1* gene region as described in Example (14-2) and inserted into pTV19ΔSph (i.e., pTV19 which was cleaved with SphI,

blunt-ended and self-ligated, and from which SphI site was deleted), which had been cleaved with BamHI and treated with BAP.

The 3.3-kb ClaI-SphI fragments isolated from the plasmid, as described in Example (8-1), which were obtained by changing the BglII site of the pROMU1 to a ClaI site and changing the HindIII site of the pROMU1 to a SphI site, respectively, by linker ligation method, were inserted into the ClaI-SphI of the obtained plasmid. The resultant plasmid was named pDOMPB1 (Fig. 15).

(15-2) Transformation

The pDOMPB1 obtained in Example (15-1) was cleaved with BamHI and transformed into the *Ogataea minuta* TK6 strain (och1Δ pep4Δ ura3Δ) and the *Ogataea minuta* TK7 strain (och1Δ pep4Δ ura3Δ ade1Δ) obtained in Example (13-2) by electric pulse method.

The *PRB1* knockout mutants were screened by subjecting the chromosomal DNAs of the obtained transformants to Southern analysis. Specifically, when cleaving the chromosomal DNAs of the host strain and the transformants with BamHI and subjecting the cleaved chromosomal DNAs to Southern analysis using the 5-kb BamHI fragment isolated from the pDOMPB1 (Fig. 15) as a probe, 5 kb band was detected in the host strain, while 8.5 kb band was detected in the knockout mutants. After culturing the knockout mutants in the YPD medium until stationary phase, a strain resistant to 5-fluoroorotic acid (5-FOA) was obtained. The chromosomal DNA of the 5-FOA resistant strain was cleaved with BamHI and again subjected to Southern analysis using the 5-kb BamHI fragment isolated from the pDOMPB1 (Fig. 15) as a probe, and a strain was selected from which the *URA3* gene was deleted and for which 5 kb band was detected. The och1Δ pep4Δ prb1Δ ura3Δ strain obtained was named *Ogataea minuta* TK8 strain, and the och1Δ pep4Δ prb1Δ ura3Δ ade1Δ strain was named *Ogataea minuta* TK9 strain.

Example 16

Cloning of *KTR1* gene of *Ogataea minuta*

The *KTR1* gene was obtained from *Ogataea minuta* IFO 10746 and its nucleotide sequence was determined.

(16-1) Preparation of probe

The amino acid sequences conserved in the *KTR* gene family from *Saccharomyces cerevisiae* (Biochim. Biophys, Acta, (1999) Vol. 1426, p326) was extracted:

H(N)YDWV(T)FLND (SEQ ID NO:59); and

YNLCHFWSNFEI (SEQ ID NO:60),

and oligonucleotides having nucleotide sequences corresponding the above amino acid sequences were synthesized as follows.

PKR5; 5'-MAYTAYGAYTGGRYNTTYYTNAAYGA-3' (SEQ ID NO:61)

PKR3; 5'-ATYTCRAARTTNSWCCARAARTGRCANARRTTRTA-3' (SEQ ID NO:62)

The primer PKR5 has a sequence complementary to the nucleotide sequences corresponding to the amino acid sequence H(N)YDWV(T)FLND, and the primer PKR3 has a sequence complementary to the nucleotide sequences corresponding to the amino acid sequence YNLCHFWSNFEI.

PCR by primers PKR5 and PKR3 was performed using the chromosomal DNA of *Ogataea minuta* IFO 10746 as a template ((94°C for 30 seconds, 50°C for 1 minute and 72°C for 1 minute) × 25 cycles). The amplified DNA fragment of approximately 0.6 kb was recovered and cloned using TOPO TA Cloning Kit. Plasmid DNA was isolated from the obtained clone and sequenced. From the nucleotide sequence analysis for 60 clones, it was confirmed that total 4 types of gene fragments existed, all of which had a high homology with the amino acid sequences of the *KTR1* gene family from *Saccharomyces cerevisiae*. One clone was selected from the 60 clones and the 0.6-kb DNA insert was recovered after EcoRI cleavage of the plasmid and separation by agarose gel electrophoresis.

(16-2) Preparation of library and screening

The chromosomal DNA of *Ogataea minuta* IFO 10746 was cleaved with different restriction enzymes and subjected to Southern analysis using the DNA fragment obtained in Example (12-1) as a probe by the method described in Example (2-2). The results suggested

that there existed the *KTR1* gene in the SacI fragment of approximately 2 kb. Then, to clone the DNA fragment, a library was prepared. The chromosomal DNA of *Ogataea minuta* was cleaved with SacI and subjected to agarose gel electrophoresis, and then the approximately 2-kb DNA fragment was recovered from the gel. The DNA fragment was ligated with SacI-cleaved and BAP-treated pUC18 and then transformed into *Escherichia coli* strain DH5 α to prepare a library.

About 4,000 clones were screened by colony hybridization using the above described DNA fragment as a probe. A clone bearing plasmid pOMKR1 was selected from the 2 positive clones obtained.

(16-3) Sequencing of nucleotide sequence

The nucleotide sequence of the SacI insert in the plasmid pOMKR1 (Fig. 16) was determined by deletion mutant and primer walking method to obtain a nucleotide sequence represented by SEQ ID NO:63.

In the nucleotide sequence of SEQ ID NO:63, there existed an open reading frame of 1,212 bp, starting at position 124 and ends at position 1,335. The homology studies between the amino acid sequence (SEQ ID NO:64) deduced from the open reading frame and the *KTR1* or *KRE2* gene product, as *KTR* family, from *Saccharomyces cerevisiae*, showed that 53% or 49% of amino acids were respectively identical between them

Example 17

Preparation of *Ogataea minuta KTR1* knockout mutant

The *KTR1* gene was disrupted by transformation using the *URA3* gene of *Ogataea minuta* as a marker.

(17-1) Preparation of *KTR1* gene disruption vector

As shown in Fig. 16, plasmid pDOMKR1 was prepared by replacing the 0.3-kb EcoRI-BglII region of the *KTR1* structural gene by the *URA3* gene. To obtain a uracil auxotrophic mutant again from *KTR1* knockout mutants, the *URA3* gene having repetitive structures before and after the structural gene was used as a marker. The plasmid pOMKR1

carrying the *KTR1* gene region as described in Example (16-2) was cleaved at HindIII-XbaI, blunt-ended, and ligated. The obtained plasmid was cleaved with EcoRI and ligated with a HindIII linker.

The 3.3-kb BglII-HindIII fragment isolated from the pROMU1 as described in Example (8-1) was inserted into the BglII-HindIII of the obtained plasmid. The resultant plasmid was named pDOMKR1 (Fig. 16).

(17-2) Transformation

The pDOMKR1 obtained in Example (17-1) was cleaved at SacI-ClaI and transformed into the *Ogataea minuta* TK8 strain (och1Δ pep4Δ prb1Δ ura3Δ) and the *Ogataea minuta* TK9 strain (och1Δ pep4Δ prb1Δ ura3Δ ade1Δ) obtained in Example (15-2), by electric pulse method.

The *KTR1* knockout mutants were screened by subjecting the chromosomal DNAs of the obtained transformants to Southern analysis. Specifically, the chromosomal DNAs of the host strain and the transformants were cleaved with SacI and subjected to Southern analysis using the 2-kb SacI fragment isolated from the pDOMKR1 (Fig. 16) as a probe. As a result, 2 kb band was detected in the host strain, while 5 kb band was detected in the knockout mutants. After culturing the knockout mutants in the YPD medium until stationary phase, a strain resistant to 5-fluoroorotic acid (5-FOA) was obtained. The chromosomal DNA of the 5-FOA resistant strain was cleaved with SacI and again subjected to Southern analysis using the 2-kb SacI fragment isolated from the pDOMKR1 (Fig. 16) as a probe, and a strain was selected from which the *URA3* gene was deleted and for which 5 kb band was detected. The och1Δ ktr1Δ pep4Δ prb1Δ ura3Δ strain obtained was named *Ogataea minuta* TK10 strain, and the och1Δ ktr1Δ pep4Δ prb1Δ ura3Δ ade1Δ strain was named *Ogataea minuta* TK11 strain.

The sensitivity of *Ogataea minuta* TK10 and *Ogataea minuta* TK11 strains to hygromycin B was examined. *Ogataea minuta* IFO 10746, a wild strain, yielded colonies on a plate containing 50 μg/ml hygromycin B, but neither *Ogataea minuta* TK10 nor *Ogataea minuta* TK11 strain yielded a colony even on a plate containing 5 μg/ml hygromycin B. It is known that sugar chain mutants of *Saccharomyces cerevisiae* have higher sensitivity to a drug

like hygromycin B than the wild strain of the same. Thus, it was presumed that these *Ogataea minuta* ktr1Δ strains had short sugar chains.

Further, in the *Ogataea minuta* ktr1Δ strains, the precipitation of cells was markedly increased just like the *Saccharomyces cerevisiae* och1Δ strain. This may show that the sugar chains of these *Ogataea minuta* ktr1Δ strains were short.

Example 18

Cloning of *MNN9* gene of *Ogataea minuta*

The *MNN9* gene was obtained from *Ogataea minuta* IFO 10746 and its nucleotide sequence was determined.

(18-1) Preparation of probe

Oligonucleotides having nucleotide sequences corresponding to the following amino acid sequences conserved in *MNN9* from *Saccharomyces cerevisiae* (GenBank accession number; L23752) and *Candida albicans* (GenBank accession number; U63642):

TSWVLWLDAD (SEQ ID NO:65); and

ETEGFAKMAK (SEQ ID NO:66)

were synthesized as follows.

PMN5; 5'-ACNWSNTGGGTNYTNTGGYTNGAYGCNGA-3' (SEQ ID NO:67)

PMN3; 5'-TTNGCCATYTTNGCRAANCCYTCNGTYTC-3' (SEQ ID NO:68)

The primer PMN5 has a sequence complementary to the nucleotide sequence corresponding to the amino acid sequence TSWVLWLDAD, and the primer PMN3 has a sequence complementary to the nucleotide sequence corresponding to the amino acid sequence ETEGFAKMAK.

PCR by primers PMN5 and PMN3 was performed using the chromosomal DNA of *Ogataea minuta* IFO 10746 as a template ((94°C for 30 seconds, 50°C for 1 minute and 72°C for 1 minute) × 25 cycles). The amplified DNA fragment of approximately 0.4 kb was recovered and cloned using TOPO TA Cloning Kit. Plasmid DNA was isolated from the obtained clone and sequenced. For a DNA insert of the plasmid, a clone was selected which had a nucleotide sequence encoding an amino acid sequence highly homologous to the amino

acid sequences for *MNN9* genes from *Saccharomyces cerevisiae* and *Candida albicans.* The 0.4-kb DNA insert was recovered after EcoRI cleavage of the plasmid and agarose gel electrophoresis.

(18-2) Preparation of library and screening

The chromosomal DNA of *Ogataea minuta* IFO 10746 was cleaved with different restriction enzymes, and subjected to Southern analysis using the DNA fragment obtained in Example (18-1) as a probe by the method described in Example (2-2). The results suggested that there existed the *MNN9* gene in the BamHI fragment of approximately 8 kb. Then, to clone the DNA fragment, a library was prepared. The chromosomal DNA of *Ogataea minuta* was cleaved with BamHI and subjected to agarose gel electrophoresis, and then the approximately 8-kb DNA fragment was recovered from the gel. The DNA fragment was ligated with BamHI-cleaved pUC118 and then transformed into *Escherichia coli* strain DH5 α to prepare a library.

About 6,000 clones were screened by colony hybridization using the above described DNA fragment as a probe. A clone bearing plasmid pOMMN9 was selected from the 2 positive clones obtained.

(18-3) Sequencing of nucleotide sequence

The nucleotide sequence of the ApaI-BglII region of the plasmid pOMMN9 (Fig. 17) was determined by deletion mutant and primer walking method to obtain a nucleotide sequence represented by SEQ ID NO:69.

In the nucleotide sequence of SEQ ID NO:69, there existed an open reading frame of 1,104 bp, starting at position 931 and ends at position 2,034. The homology studies between the amino acid sequence (SEQ ID NO:70) deduced from the open reading frame and the *MNN9* gene product from *Saccharomyces cerevisiae* or *Candida albicans* showed that 59% or 62% of amino acids were respectively identical between them.

Example 19

Preparation of *Ogataea minuta MNN9* knockout mutant

The *MNN9* gene was disrupted by transformation using the *URA3* gene of *Ogataea minuta* as a marker.

(19-1) Preparation of *MNN9* Disruption Vector

As shown in Fig. 17, plasmid pDOMN9 was prepared by replacing the approximately 1-kb SaI-BglII region of the *MNN9* structural gene by the *URA3* gene. To obtain a uracil auxotrophic mutant again from *MNN9* knockout mutants, the *URA3* gene having repetitive structures before and after the structural gene was used as a marker. The 1.2-kb ApaI-SalI fragment isolated from the plasmid pOMMN9-1 having the *MNN9* gene region described in Example 18 was inserted into the ApaI-SalI of the pBluescript II SK-. The 2.2-kb NheI-BglII fragments isolated from the plasmid pOMMN9-1 and the 3.3 kb BglII-HindIII fragment isolated from the pROMU1 described in Example (8-1) were inserted into the XbaI-HindIII of the obtained plasmid. The resultant plasmid was named pDOMN9 (Fig. 17).

(19-2) Transformation

The pDOMN9 obtained in Example (19-1) was cleaved with ApaI and transformed into the *Ogataea minuta* TK8 strain (och1Δ pep4Δ prb1Δ ura3Δ), the *Ogataea minuta* TK9 strain (och1Δ pep4Δ prb1Δ ura3Δ ade1Δ) obtained in Example (15-2) and the *Ogataea minuta* TK10 strain (och1Δ ktr1Δ pep4Δ prb1Δ ura3Δ), the *Ogataea minuta* TK11 strain (och1Δ ktr1Δ pep4Δ prb1Δ ura3Δ ade1Δ) obtained in Example (17-2), by electric pulse method.

The *MNN9* knockout mutants were screened by subjecting the chromosomal DNAs of the obtained transformants to Southern analysis. Specifically, the chromosomal DNAs of the host strain and the transformants were cleaved with ApaI and BglII and subjected to Southern analysis using the 1.2-kb ApaI-SalI fragment isolated from the pOMMN9-1 (Figure 17) as a probe. As a result, a band was detected at 2.2 kb in the host strain, while a band at 5.5 kb in the knockout mutants. After culturing the knockout mutants on the YPD medium until stationary phase, a strain resistant to 5-fluoroorotic acid (5-FOA) was obtained. PCR by primers DMN5; 5'-AGATGAGGTGATTCCACGTAATTTGCCAGC-3' (SEQ ID NO:71) and DMN3; 5'-TTTTGATTGTCATCTATTTCGCACACCCTG-3' (SEQ ID NO:72) was

performed using the chromosomal DNA of the 5-FOA resistant strain as a template ((94°C for 30 seconds, 60°C for 1 minute and 72°C for 1 minute) × 25 cycles). As a result, a 1 kb amplified DNA fragment was detected in the strain from which the URA3 gene was deleted. The och1Δ mnn9Δ pep4Δ prb1Δ ura3Δ strain obtained was named *Ogataea minuta* TK12 strain, the och1Δ mnn9Δ pep4Δ prb1Δ ura3Δ ade1Δ strain *Ogataea minuta* TK13 strain, the och1Δ ktr1Δ mn9Δ pep4Δ prb1Δ ura3Δ strain was named *Ogataea minuta* TK14 strain, and the och1Δ ktr1Δ mnn9Δ pep4Δ prb1Δ ura3Δ ade1Δ strain was named *Ogataea minuta* TK15 strain.

The sensitivity of the *Ogataea minuta* TK14 and *Ogataea minuta* TK15 strains to hygromycin B was examined. *Ogataea minuta* IFO 10746, a wild strain, yielded colonies on a plate containing 50 μg/ml hygromycin B as described in Example (17-2), but neither *Ogataea minuta* TK12 nor *Ogataea minuta* TK13 strain yielded a colony even on a plate containing 20 μg/ml hygromycin B. Thus, it was presumed that these *Ogataea minuta* mnn9Δ strains had short sugar chains.

Example 20

Cloning of alcohol oxidase (*AOX1*) gene of *Ogataea minuta*

The *AOX1* gene was obtained from *Ogataea minuta* IFO 10746 and its nucleotide sequence was determined.

(20-1) Preparation of probe

Oligonucleotides having nucleotide sequences corresponding to the following amino acid sequences conserved in alcohol oxidase from *Pichia pastoris* (GenBank accession number; U96967, U96968) and *Candida boidinii* (GenBank accession number; Q00922):

GGGSSINFMMYT (SEQ ID NO:73); and

DMWPMVWAYK (SEQ ID NO:74)

were synthesized as follows.

PAX5; 5'-GGNGGNGGNWSNWSNATHAAYTTYATGATGTAYAC-3' (SEQ ID NO:75)

PAX3; 5'-TTRTANGCCCANACCATNGGCCACATRTC-3' (SEQ ID NO:76)

The primer PAX5 has a sequence complementary to the nucleotide sequence corresponding to the amino acid sequence GGGSSINFMMYT, and the primer PAX3 has a sequence complementary to the nucleotide sequence corresponding to the amino acid sequence DMWPMVWAYK.

PCR by primers PAX5 and PAX3 was performed using the chromosomal DNA of *Ogataea minuta* IFO 10746 as a template ((94°C for 30 seconds, 50°C for 1 minute and 72°C for 1 minute) × 25 cycles). The amplified DNA fragment of approximately 1.1 kb was recovered and cloned using TOPO TA Cloning Kit. Plasmid DNA was isolated from the obtained clone and sequenced. For a DNA insert of the plasmid, a clone was selected which had a nucleotide sequence encoding an amino acid sequence highly homologous to the amino acid sequences for alcohol oxidase genes from *Pichia pastoris* and *Candida boidinii.* The 1.1-kb DNA insert was recovered after EcoRI cleavage of the plasmid and agarose gel electrophoresis.

(20-2) Preparation of library and screening

The chromosomal DNA of *Ogataea minuta* IFO 10746 was cleaved with different restriction enzymes, and subjected to Southern analysis using the DNA fragment obtained in Example (20-1) as a probe by the method described in Example (2-2). The results suggested that there existed *AOX1* gene in the HindIII fragment of approximately 8 kb. Then, to clone the DNA fragment, a library was prepared. The chromosomal DNA of *Ogataea minuta* was cleaved with HindIII and subjected to agarose gel electrophoresis, and then the approximately 6-kb DNA fragment was recovered from the gel. The DNA fragment was ligated with HindIII-cleaved pUC118 and then transformed into *Escherichia coli* strain DH5 α to prepare a library.

About 6,000 clones were screened by colony hybridization using the above described DNA fragment as a probe. A clone bearing plasmid pOMAX1 was selected from the 6 positive clones obtained.

(20-3) Sequencing of nucleotide sequence

The nucleotide sequence of the HindIII-SmaI region of the plasmid pOMAX1 (Fig. 18) was determined by deletion mutant and primer walking method to obtain a nucleotide sequence represented by SEQ ID NO:77.

In the nucleotide sequence of SEQ ID NO:77 there existed an open reading frame of 1,992 bp, starting at position 2,349 and ends at position 4,340. The homology studies between the amino acid sequence (SEQ ID NO:78) deduced from the open reading frame and the alcohol oxidase from *Pichia pastoris* or *Candida boidinii* showed that 72% or 74% of amino acids were respectively identical between them.

Example 21

Construction of heterologous gene expression plasmid using *AOX1* gene promoter and terminator

(21-1) Construction of expression cassette using *AOX1* gene promoter and terminator

An expression cassette was constructed for transferring foreign genes between the *Ogataea minuta AOX1* gene promoter (SEQ ID NO:79) and terminator (SEQ ID NO:80). To transfer XbaI, SmaI and BamHI sites between the *AOX1* gene promoter and terminator, the following primers were synthesized:

OAP5; 5'-CTGCAGCCCCTTCTGTTTTTCTTTTGACGG-3' (SEQ ID NO:81)

OAP3;5'-CCCCCGGATCCAGGAACCCGGGAACAGAATCTAGATTTTTTCGTAAGT CGTAAGTCGTAACAGAACACAAGAGTCTTTGAACAAGTTGAG-3' (SEQ ID NO:82)

OAT5;5'-CCCCCCCGGATCCGAGACGGTGCCCGACTCTTGTTCAATTCTTTTGG-3' (SEQ ID NO:83)

OAT3; 5'-CCCATAATGGTACCGTTAGTGGTACGGGCAGTC-3' (SEQ ID NO:84)

PCR by primers OAP5 and OAP3 ((94°C for 30 seconds, 55°C for 1 minute and 72°C for 1 minute) × 20 cycles), and PCR by primers OAT5 and OAT3 ((94°C for 30 seconds, 55°C for 1 minute and 72°C for 1 minute) × 20 cycles) were performed using the pOMAX1 shown in Fig. 18 as a template. The amplified DNA fragments of 0.5 kb and 0.8 kb were recovered and cloned using TOPO TA Cloning Kit. The nucleotide sequences of DNA inserts were determined, and then clones having correct nucleotide sequences were selected. The DNA

inserts of 0.5 kb and 0.8 kb were isolated as PstI-BamHII fragment and BamHI-KpnI fragment, respectively. The above described 0.5-kb PstI-BamHII fragment was inserted into the PstI-BamHII of the pOMAX1. Then, the 0.8-kb BamHI-KpnI fragment was inserted into the BamHI-KpnI of the obtained plasmid. The resultant plasmid was named pOMAXPT1 (Fig. 18).

The pOMAXPT1 had an expression cassette controlled by the *AOX1* promoter and terminator that allowed foreign genes to be transferred at the XbaI, SmaI and BamHI sites.

(21-2) Construction of heterologous gene expression plasmid using *AOX1* gene promoter and terminator, and using *URA3* gene as a selectable marker

The 3.1-kb Bg1II-HindIII fragment containing the *Ogataea minuta URA3* gene and isolated from the pOMUR1 described in Example (5-2) was inserted into the BamHI-HindIII of pUC19. The obtained plasmid was named pOMUR5 (Fig. 18). The pOMUR5 was cleaved with StyI and SacI and blunt-ended, and ApaI linkers were then inserted thereinto. The obtained plasmid was named pOMUR6. The pOMUR6 was cleaved with XbaI and blunt-ended, and ligated. The obtained plasmid was named pOMUR-X. The pOMUR-X was cleaved with SalI and blunt-ended, and a NotI linker was inserted thereinto.

The resultant plasmid was named pOMUR-XN. The 3.1-kb HindIII-KpnI fragment containing the expression cassette controlled by the *Ogataea minuta AOX1* promoter and terminator which was isolated from the pOMAXPT1 as described in Example (21-1), was inserted into the HindIII-KpnI of the pOMUR-XN. The obtained plasmid was named pOMex1U (Fig. 18).

The pOMex1U was cleaved with BglII and blunt-ended, and a NotI linker was inserted thereinto. The obtained plasmid was named pOMex1U-NO (Fig. 18). The 3.1-kb HindIII-KpnI fragment containing the expression controlled by the *Ogataea minuta AOX1* gene promoter and terminator which was isolated from the pOMex1U-NO, was inserted into the HindIII-KpnI of the pOMUR-X. The resultant plasmid was named pOMex2U (Fig.18).

(21-3) Construction of heterologous gene expression plasmid using *AOX1* gene promoter and terminator, and using G418 resistant gene as a selectable marker

The pOMKmR1, which comprised the G418 resistant gene expression cassette controlled by the *GAP* gene promoter and terminator described in Example 4, was cleaved with PstI and blunt-ended, and an ApaI linker was inserted thereinto. The G418 resistant gene expression cassette was isolated, as a 2.3-kb ApaI-KpnI fragment, from the obtained plasmid and inserted into the ApaI-KpnI of the POMex1U-NO described in Example (21-2). The resultant plasmid was named pOMex3G (Fig. 18).

(21-4) Construction of heterologous gene expression plasmid using *AOX1* gene promoter and terminator, and using *ADE1* gene as a selectable marker

A plasmid was prepared by cleaving with SmaI the pOMAD1, which contained the *ADE1* gene described in Example 7, transferring an ApaI linker, cleaving with EcoRV, transferring a KpnI linker, cleaving with BglII, blunt-ending, and transferring a NotI linker. The *ADE1* gene expression cassette was isolated, as a 3.1-kb ApaI-KpnI fragment, from the obtained plasmid, and inserted into the ApaI-KpnI containing the expression cassette controlled by the *Ogataea minuta AOX1* gene promoter and terminator which was obtained by ApaI-KpnI from the pOMex1U. The resultant plasmid was named pOMex4A (Fig. 18).

(21-5) Construction of heterologous gene expression plasmid using *AOX1* gene promoter and terminator, and using hygromycin B resistant gene as a selectable marker

To perform transformation by the selection of antibiotic hygromycin B resistance, a plasmid containing the hygromycin B resistant gene (hygromycin B phosphotransferase gene) expression cassette was constructed.

To isolate the hygromycin B resistant gene, the following primers were synthesized:
HGP5; 5'-GTCGACATGAAAAAGCCTGAACTCACCGC-3' (SEQ ID NO:85); and
HGP3; 5'-ACTAGTCTATTCCTTTGCCCTCGGACG-3' (SEQ ID NO:86).

PCR by primers HGP5 and HGP3 was performed using the plasmid pGARH containing the hygromycin B resistant gene (Applied Environ. Microbiol., Vol. 64 (1998)

p2676) as a template ((94°C for 30 seconds, 50°C for 1 minute and 72°C for 1 minute) × 20 cycles). The 1.0 kb amplified DNA fragment was recovered and cloned using TOPO TA Cloning Kit.

The nucleotide sequence of the DNA insert was determined, and a clone having the correct nucleotide sequence was selected. The 1.0 kb DNA insert was isolated as a SalI-EcoT22II fragment and inserted into the SalI-EcoT22I of the pOMGP4 constructed in Example 3. The obtained plasmid was named pOMHGR1. The obtained plasmid was cleaved with HindIII and blunt-ended, and an ApaI linker was inserted thereinto. The hygromycin B resistant gene expression cassette was isolated, as a 3.0-kb ApaI-KpnI fragment, from the obtained plasmid, and then inserted into the ApaI-KpnI of the pOMex1U-NO described in Example 21-2. The resultant plasmid was named pOMex5H (Fig. 18).

Example 22

Construction of heterologous gene expression plasmid using *GAP* gene promoter and terminator, and using *URA3* gene as a selectable maker

The gene expression cassette using the *GAP* gene promoter and terminator, as described in Example 3, was isolated as a 2.0-kb HindIII-KpnI, and then inserted into the HindIII-KpnI of each of the pOMUR-XN described in Example (21-2) and the pOMex4A described in Example (21-4) (where pOMex4A was a fragment comprising pUC19-ADE1). The obtained plasmids were named pOMexGP1U and pOMexGP4A, respectively (Fig. 18).

Example 23

Construction of *Aspergillus saitoi*-derived α-1,2-mannosidase expression plasmid using *AOX1* gene promoter and terminator

Example 11 suggested that expression of α-1,2-mannosidase in the *Ogataea minuta* Δoch1 strain enabled the preparation of a Man5 producing yeast. So, *Ogataea minuta* Δoch1 strain in which α-1,2-mannosidase was expressed was prepared. The *Aspergillus saitoi*-derived α-1,2-mannosidase gene, which comprised a signal sequence of asperginopepsin I (apnS) at the amino terminus and a yeast endoplasmic reticulum (ER)

retention signal (HDEL) at the carboxyl terminus (J. Biol. Chem., *273* (1998) 26298), was used for expression. PCR by the primers:

5'-GGGGGGTCGACATGGTGGTCTTCAGCAAAACCGCTGCCC-3' (SEQ ID NO:87); and

5'-GGGGGGCGGCCGCGTGATGTTGAGGTTGTTGTACGGAACCCCC-3' (SEQ ID NO:88)

was performed using the plasmid pGAMH1 comprising the above described gene as a template ((94°C for 30 seconds, 50°C for 1 minute and 72°C for 30 seconds) × 20 cycles). The approximately 0.5-kb DNA fragment 5'-upstream of the amplified α-1,2-mannosidase gene was recovered, cleaved with SalI and NotI, and inserted into the SalI-NotI of the pBluescript II SK-. The nucleotide sequence of the DNA insert was determined and a clone comprising the correct nucleotide sequence was selected. The 1.2-kb BglII-NotI fragment downstream of the BglII site in the α-1,2-mannosidase gene isolated from the pGAMH1 was inserted into the BglII-NotI of the obtained plasmid. This plasmid was named paMSN. The paMSN was cleaved with SalI and blunt-ended, and an XbaI linker was inserted thereinto. This plasmid was named paMXN. Separately, the paMSN was cleaved with NotI and blunt-ended, and a BamHI linker was inserted thereinto. The resultant plasmid was named paMSB. The 0.4-kb XbaI-BglII fragment upstream of the α-1,2-mannosidase gene isolated after cleaving the paMXN with XbaI-ApaI, and the 1.1-kb ApaI-BamHI fragment downstream of the α-1,2-mannosidase gene isolated after cleaving the paMSB with ApaI-BamHI, were inserted into the XbaI-BamHII of the pOMex1U described in Example (21-2) and of the pOMex3G described in Example (21-3), respectively, by three points ligation. The obtained plasmids were named pOMaM1U and pOMaM3G, respectively.

Example 24

Preparation of *Aspergillus saitoi*-derived α-1,2-mannosidase gene expressing *Ogataea minuta* Δoch1 strain and sugar chain analysis of same

The pOMaM1U obtained in Example 23 was cleaved with NotI, and the *Ogataea minuta* TK3-A strain (och1Δ ura3Δ) obtained in Example (10-2) was transformed with it.

The intracellular α-1,2-mannosidase activity of the obtained transformant was measured. The transformants cultured in the BYPM medium (0.67% yeast nitrogen base, 1% yeast extract, 2% polypeptone, 100 mM potassium phosphate buffer pH 6.0, 0.5% methanol) were harvested and suspended in 0.1 M sodium acetate buffer pH 5.0 containing 1% Triton X100 and 1mM PMSF, then the cells were disrupted with glass beads to obtain a cell extract. The extract was appropriately diluted, 20 pmol of Man6b sugar chain (TAKARA SHUZO CO., LTD., Japan) was added, and the mixture was incubated for reaction at 37°C for 10-60 minutes. After the incubation, the mixture was boiled to inactivate the enzyme and subjected to HPLC to analyze the produced Man5 sugar chain. The HPLC conditions were as follows.

Column: TSK-Gel ODS 80TM (6 × 150 mm, TOSOH CORPORATION, Japan)

Column temperature: 50°C

Flow rate: 1.2 ml

Elution conditions: A: 100 mM ammonium acetate pH 6.0

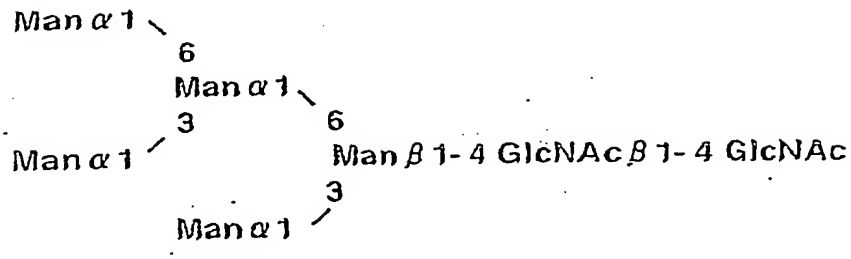
B: 100 mM ammonium acetate pH 6.0 + 0.15% butanol

Linear gradient of 0 minute A = 70% and 12 minutes A = 0%

A yeast strain having the highest α-1,2-mannosidase activity was selected and named *Ogataea minuta* TK3-A-MU1 strain. The yeast strain was cultured again in the BYPM medium, and the structure of the sugar chain of cell surface mannan proteins was analyzed. The preparation of PA-oligosaccharides was carried out in accordance with the method described in Example 1. And HPLC analysis was performed by the method described in Example 11.

The results are shown in Fig. 19. The size analysis by normal phase column revealed that the *Ogataea minuta* TK3-A-MU1 strain mainly produced Man5GlcNAc2. The structure analysis by reverse phase column revealed that the Man5GlcNAc2 was the sugar chain of the following structural formula 2:

Structural Formula 2



which sugar chain was consistent with the human-type, high mannose-type sugar chain, and precursor of hybrid type or complex type sugar chains.

Example 25

Construction of *Saccharomyces cerevisiae*-derived invertase expression plasmid using *AOX1* gene promoter and terminator

Invertase (*SUC2*) gene of *Saccharomyces cerevisiae* (GenBank accession number; V01311) was obtained by PCR. PCR by the primers:

5'-GGGGACTAGTATGCTTTTGCAAGCTTTCCTTTTCCTTTTG-3' (SEQ ID NO:89); and

5'-CCCCAGATCTTATTTTACTTCCCTTACTTGGAACTTGTC-3' (SEQ ID NO:90)

was performed using the chromosomal DNA of *Saccharomyces cerevisiae* S288C strain as a template ((94°C for 30 seconds, 50°C for 1 minute and 72°C for 1.5 minute) × 20 cycles). The amplified DNA fragment of approximately 1.4 kb was recovered, cleaved with SpeI and BglII, and inserted into the Xbal-BamHI of the pOMex1U described in Example (21-2) and of the pOMex3G described in Example (21-3). The obtained plasmids were named pOMIV1U and pOMIV3G, respectively.

Example 26

Transferring of *Saccharomyces cerevisiae*-derived invertase gene into *Aspergillus saitoi*-derived α-1,2-mannosidase gene expressing *Ogataea minuta OCH1* knockout mutant and expression of same

The pOMIV3G obtained in Example 25 was cleaved with NotI and transferred into the *Ogataea minuta* TK3-A-MU1 strain described in Example 24. The transformant was cultured in the BYPM medium (0.67% yeast nitrogen base, 1% yeast extract, 2% polypeptone,

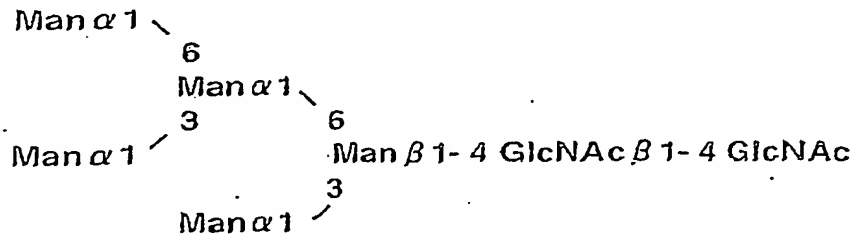
100 mM potassium phosphate buffer pH 6.0, 0.5% methanol). The culture was centrifuged and the resultant supernatant was assayed for invertase activity by the following procedures. Specifically, 2 μl of appropriately diluted culture supernatant and 200 μl of 100 mM sodium acetate buffer (pH 5.0) containing 2% sucrose were mixed together and incubated at 37°C for 10-30 minutes, and 500 μl of Glucose-Test Wako (Wako Pure Chemical Industries, Ltd., Japan) was added to 2 μl of the reaction mixture to develop color. An absorbance based on free glucose generated by invertase was measured at 505 nm. The most productive yeast strain *Ogataea minuta* TK3-A-MU-IVG1 strain produced about 600 mg invertase/l medium, and the invertase was most part of proteins in the culture supernatant.

Example 27

Structure analysis of sugar chain of *Saccharomyces cerevisiae*-derived invertase secreted by the strain prepared in Example 26

The culture supernatant of the *Ogataea minuta* TK3-A-MU-IVG1 strain obtained in Example 26 was concentrated by ultrafiltration using Amicon YM76 membrane (Amicon), desalted, and subjected to an anion exchange column chromatography (Q-Sepharose FF, Amersham Pharmacia Biotech) to purify invertase fractions. The fractions were freeze-dried and PA-N-linked sugar chain was prepared by the method described in Example 1. The analysis by HPLC was performed by the method described in Example 11. The results are shown in Fig. 20. The results of the size analysis by amide column revealed that 90% or more sugar chains of the invertase was composed of Man5GlcNAc2. The structure analysis by reverse phase column showed that the Man5GlcNAc2 was the sugar chain represented by the structural formula 2 described in Example 24:

Structural Formula 2



This sugar chain was consistent with the Man5 type, high mannose type sugar chain, which is a precursor of hybrid type or complex type sugar chain.

Example 28

Preparation of human antibody gene-transferred *Ogataea minuta OCH1* knockout mutant, transfer and expression of *Aspergillus saitoi*-derived α-1,2-mannosidase gene in the mutant, and production of human antibody using same

Anti-human G-CSF antibody gene was transferred into the *Ogataea minuta* TK9 strain (och1Δ pep4Δ prb1Δ ura3Δ ade1Δ) obtained in Example (15-2).

Anti-human G-CSF antibody producing hybridoma was obtained by producing a mouse producing anti-human G-CSF antibodies using human G-CSF as an antigen in accordance with the method by Tomiduka et al. (Proc. Natl. Acad. Sci. U.S.A. 97(2), 722-7 (2000)), removing the spleen from the mouse by conventional procedure (Muramatsu et al., Jikken Seibutsugaku Koza, Vol. 14, pp.348-364), and fusing the B cells with a mouse myeloma. The antibody gene was obtained from the hybridoma by the method described by Welschof, M et al. (J. Immunol. Methods. 179 (2), 203 -14 (1995)).

XbaI linker and BamHI linker were added at the N-terminus and the C-terminus, respectively, of each of the anti-G-CSF light chain gene (SEQ ID NO:91; the coded amino acid sequence, SEQ ID NO:92) and anti-G-CSF heavy chain gene (SEQ ID NO:93, the coded amino acid sequence, SEQ ID NO:94). Subsequently, the light chain gene was transferred at the XbaI-BamHI site of the pOMex4A described in Example (21-4) while the heavy chain gene at the XbaI-BamHI site of the pOMex3G described in Example (21-3), respectively. Each of the constructed expression vectors was cleaved with NotI, and the *Ogataea minuta* TK9 strain was in turn transformed. The obtained transformants were cultured in the BYPMG medium (0.67% yeast nitrogen base, 1% yeast extract, 2% polypeptone, 100 mM potassium phosphate buffer pH 6.0, 0.1% methanol, 0.2% glycerol) at 20°C for 72 hours, and then centrifuged. The culture supernatant was subjected to Western analysis using a

horseradish peroxidase labeled anti-human IgG sheep antibody (Amersham Pharmacia Biotech). First, 100 μl of the culture supernatant was concentrated through Microcon YM30 membrane and subjected to SDS-PAGE. Then, the electrophoresed proteins were blotted on PVDF membrane (Immobilon, Millipore), which membrane was then blocked over 1 hour using Block Ace (Dainippon Pharmaceutical Co., Ltd., Japan). Proteins on the membrane were incubated for 1 hour in TBS solution (Tris buffer containing 0.15 M NaCl) containing the horseradish peroxidase labeled anti-human IgG sheep antibody (1000:1 dilution), and unbound antibodies were washed out with TBS containing 0.04% Tween 20. The detection of signal was carried out using Super Signal WestDura (Pierce). Thus, the transformant producing the antibody in the culture supernatant was selected. The *Ogataea minuta* TK9-derived antibody producing strain was named *Ogataea minuta* TK9-IgB1.

Then, the *Aspergillus saitoi*-derived α-1,2-mannosidase gene was transferred into the *Ogataea minuta* TK9-IgB1 strain. After transformation, α-1,2-mannosidase expressing strain was selected from the obtained transformants by the method described in Example 24 using the plasmid pOMaM1U prepared in Example 23. The resultant strain was named *Ogataea minuta* TK9-IgB-aM. This strain was cultured in the BYPMG medium at 20°C for 72 hours and centrifuged. The culture supernatant obtained by the centrifugation was subjected to Western analysis.

The results are shown in Fig. 21. The results revealed that the *Ogataea minuta* TK9-IgB-aM strain produced both antibody heavy chains and light chains, although part of the antibody heavy chains was degraded.

Further, the culture supernatant of the *Ogataea minuta* TK9-IgB-aM strain was concentrated by ultrafiltration using Amicon YM76 membrane (Amicon), desalted, and subjected to Protein A column chromatography (Hi-Trap ProteinA HP, Amersham Pharmacia Biotech) to purify the antibody fractions through the elution with glycine-HCl, pH 3.0 (Fig. 22). To detect the binding of the antibody to G-CSF as the antigen, Western analysis was performed. The analysis was done in accordance with the above described procedures using the purified antibody as a primary antibody and the horseradish peroxidase labeled anti-human IgG sheep antibody as a secondary antibody. The results are shown in Fig. 23. The results

revealed that the antibody produced by the *Ogataea minuta* TK9-IgB1 strain bound to G-CSF as the antigen.

Example 29

Structure analysis of sugar chains of human antibody produced by the strains prepared in Example 28

The purified antibodies produced using the *Ogataea minuta* TK9-IgB-aM strain and the *Ogataea minuta* TK9-IgB strain as shown in Example 28 were dialyzed and freeze-dried. PA-N-linked sugar chains were prepared by the method described in Example 11 and subjected to size analysis by normal phase column. The results are shown in Fig. 24. The results revealed that the sugar chain of the antibody produced by the *Ogataea minuta* TK9-IgB strain was composed mainly of $Man_7GlcNAc_2$, while the sugar chain of the antibody produced by the *Ogataea minuta* TK9-IgB-aM strain was composed mainly of $Man_5GlcNAc_2$, which was a mammalian type, high mannose type sugar chain. The results indicated that 80% or more sugar chains were composed of $Man_5GlcNAc_2$.

Example 30

Cloning of *HIS3* (imidazoleglycerol phosphate dehydratase) gene from *Ogataea minuta*

The *HIS3* gene was obtained from *Ogataea minuta* IFO 10746 strain, and its nucleotide sequence was determined.

(30-1) Preparation of probe

Oligonucleotides having nucleotide sequences corresponding to the amino acid sequences conserved in *HIS3* gene products from *Saccharomyces cerevisiae* (Accession number; CAA27003) and *Pichia pastoris* (Accession number; Q92447):

VGFLDHM (SEQ ID NO:95); and

PSTKGVL (SEQ ID NO:96)

were synthesized as follows.

PHI5; 5'-TNGGNTTYYTNGAYCAYATG-3' (SEQ ID NO:97)

PHI3; 5'-ARNACNCCYTTNGTNSWNGG-3' (SEQ ID NO:98)

The primer PHI5 has a sequence complementary to the nucleotide sequence corresponding to the amino acid sequence VGFLDHM, and the primer PHI3 has a sequence complementary to the nucleotide sequence corresponding to the amino acid sequence PSTKGVL.

PCR by primers PHI5 and PHI3 was performed using the chromosomal DNA of *Ogataea minuta* IFO 10746 strain as a template ((94°C for 30 seconds, 50°C for 30 seconds and 72°C for 1 minute) × 25 cycles). The amplified DNA fragment of approximately 0.5 kb was recovered and cloned using TOPO TA Cloning Kit. Plasmid DNA was isolated from the obtained clone and sequenced. For a DNA insert of the plasmid, a clone was selected which had a nucleotide sequence encoding an amino acid sequence highly homologous to the amino acid sequences of *HIS3* gene products from *Saccharomyces cerevisiae* and *Pichia pastoris*. The 0.5-kb DNA insert was recovered after EcoRI digestion of the plasmid and agarose gel electrophoresis.

(30-2) Preparation of library and screening

The chromosomal DNA of *Ogataea minuta* IFO 10746 was cleaved with different restriction enzymes, and subjected to Southern analysis using the DNA fragment obtained in Example (30-1) as a probe by the method described in Example (2-2). The results indicated that there existed the *HIS3* gene in the PstI fragment of approximately 4 kb. Then, to clone the DNA fragment, a library was constructed. The chromosomal DNA of *Ogataea minuta* was cleaved with PstI and subjected to agarose gel electrophoresis, and then the approximately 4-kb DNA fragment was recovered from the gel. The recovered DNA fragment was ligated with PstI-cleaved and BAP-treated pUC118 and then transformed into *Escherichia coli* DH5 α strains to prepare a library.

About 2,000 clones were screened by colony hybridization using the above described DNA fragment as a probe. A clone bearing plasmid pOMHI1 was selected from the 4 positive clones obtained.

(30-3) Sequencing of nucleotide sequence

The nucleotide sequence of the PstI-PstI region of the plasmid pOMHI1 (Fig. 25) was determined by primer walking method to obtain a nucleotide sequence represented by SEQ ID NO:99.

In the nucleotide sequence of SEQ ID O: 99, there existed an open reading frame of 714 bp, starting at position 1,839 and ends at position 2,552. The homology studies between the amino acid sequence (SEQ ID NO:100) deduced from the open reading frame and the *HIS3* gene product from *Saccharomyces cerevisiae* or *Pichia pastoris* showed that 73% or 71% of amino acids were respectively identical between them.

Example 31

Preparation of *Ogataea minuta HIS3* knockout mutant

The *HIS3* gene was disrupted by transformation using the *Ogataea minuta URA3* gene as a marker.

(31-1) Preparation of *HIS3* gene disruption vector

As shown in Fig. 25, plasmid pDOMHI1 was prepared by replacing the approximately 70 bp region of the *HIS3* structural gene by the *URA3* gene.

The plasmid pROMU1 described in Example 8-1 was cleaved with Bg1II, blunt-ended, and ligated with an EcoT22I linker. The obtained plasmid was named pROMUHT.

The plasmid pOMHI1 containing the *HIS3* gene region and described in Example (30-3) was cleaved with PflMI, blunt-ended, and ligated with an EcoT22I linker. The obtained plasmid was named pOMHI2. This plasmid was then cleaved with EcoRI and SalI and ligated with the EcoRI- and SalI-cleaved pBluescript II KS+. The obtained plasmid was named pOMHI3. The pOMHI3 was cleaved with BtgI, blunt-ended, and ligated with a HindIII linker. The obtained plasmid was named pOMHI4. The 3.3-kb EcoT22I-HindIII fragment isolated from the pROMUHT was inserted into the EcoT22I-HindIII of the obtained plasmid. The resultant plasmid was named pDOMHI1 (Fig. 25).

(31-2) Transformation

The pDOMHI1 obtained in Example (30-2) was cleaved with BamHI and XhoI and transformed into the *Ogataea minuta* TK11 strain (och1Δ ktrlΔ pep4Δ prb1Δ ura3Δ adelΔ) obtained in Example (17-2) by electric pulse method. To confirm that the *HIS3* gene was disrupted, the following primers were synthesized (see Fig. 26 with regard to the position of each primer):

DHI5; 5'-.GGCCCAATAGTAGATATCCC-3' (SEQ ID NO:101)

DHI3; 5'-CACGGCCCGTGTAGCTCGTGG-3' (SEQ ID NO:102)

PCR by primers DHI5 and DHI3 was performed using the chromosomal DNA isolated from the transformant as a template ((94°C for 30 seconds, 60°C for 1 minute and 72°C for 2 minutes) × 25 cycles). As shown in Fig. 26, a 4.6 kb amplified DNA fragment was detected in the strain whose *HIS3* locus had the plasmid integrated thereinto. The selected strain was cultured on the YPD medium until stationary phase and a strain resistant to 5-fluoroorotic acid (5-FOA) was obtained. PCR by primers DHI5 and DHI3 was performed using the chromosomal DNA of the 5-FOA resistant strain as a template ((94°C for 30 seconds, 60°C for 1 minute and 72°C for 3 minutes) × 25 cycles). As shown in Fig. 26, in the strain from which the *URA3* gene was deleted, a 2 kb amplified DNA fragment was detected. This och1Δ ktrlΔ pep4Δ prb1Δ ura3Δ adelΔ his3Δ strain was named *Ogataea minuta* YK1.

Example 32

Construction of heterologous gene expression plasmid using *AOX1* gene promoter and terminator, and *HIS3* gene as a selectable marker

A plasmid was prepared by the steps of: cleaving with SacI the pOMHI1 containing the *HIS3* gene as described in Example (30-3); blunt-ending; transferring an ApaI; cleaving with NcoI; blunt-ending; transferring a KpnI linker; cleaving with EcoRI; blunt-ending; and transferring a NotI linker. The *HIS3* gene expression cassette was isolated, as a 2.6-kb ApaI-KpnI fragment, from the obtained plasmid, and inserted into the ApaI-KpnI of the POMex1U. The resultant plasmid was named pOMex6HS (Fig. 32).

The approximately 1.4-kb SpeI-BglII fragment comprising *Saccharomyces cerevisiae*-derived invertase gene, which was prepared in Example 25, was inserted into the

XbaI-BamHI of the pOMex6HS to prepare pOMIV6HS. This plasmid was cleaved with NotI and transferred into the *Ogataea minuta* YK1 strain described in Example (31-2). The transformants were cultured in the BYPM medium (0.67% yeast nitrogen base, 1% yeast extract, 2% polypeptone, 100 mM potassium phosphate buffer pH 6.0, 0.5% methanol). The culture was centrifuged, and invertase activity was measured for the supernatant by the following procedures. Specifically, 2 μl of the culture supernatant appropriately diluted and 200 μl of 100 mM sodium acetate buffer (pH 5.0) containing 2% sucrose were mixed and incubated at 37°C for 10-30 minutes, and then 500 μl of Glucose-Test Wako (Wako Pure Chemical Industries, Ltd., Japan) was added to the reaction mixture to develop color. An absorbance based on free glucose generated by invertase was measured at 505 nm. In the yeast strain *Ogataea minuta* YK1-IVH1, a significant amount of invertase was produced in the medium.

Example 33

Cloning of *LEU2* (3-isopropylmalate dehydrogenase) gene from *Ogataea minuta*

The *LEU2* gene was obtained from *Ogataea minuta* strain IFO 10746, and its nucleotide sequence was determined.

(33-1) Preparation of probe

Oligonucleotides having nucleotide sequences corresponding to the amino acid sequences conserved in *LEU2* gene products from *Saccharomyces cerevisiae* (Accession number; CAA27459) and *Pichia angusta* (P34733):

AVGGPKWG (SEQ ID NO:103); and

AAMMLKL (SEQ ID NO:104)

were synthesized as follows.

PLE5; 5'-GCNGTNGGNGGNCCNAARTGGGG-3' (SEQ ID NO:105)

PLE3; 5'-NARYTTNARCATCATNGCNGC-3' (SEQ ID NO:106)

The primer PLE5 has a sequence complementary to the nucleotide sequence corresponding to the amino acid sequence AVGGPKWG, and the primer PLE3 has a sequence

complementary to the nucleotide sequence corresponding to the amino acid sequence AAMMLKL.

PCR by primers PLE5 and PLE3 was performed using the chromosomal DNA of *Ogataea minuta* IFO 10746 as a template ((94°C for 30 seconds, 50°C for 1 minute and 72°C for 1 minute) × 25 cycles). The amplified DNA fragment of approximately 0.7 kb was recovered and cloned using TOPO TA Cloning Kit. Plasmid DNA was isolated from the obtained clone and sequenced. For a DNA insert of the plasmid, a clone was selected which had a nucleotide sequence encoding an amino acid sequence highly homologous to the amino acid sequence of *LEU2* gene products from *Saccharomyces cerevisiae* and *Pichia angusta.* The 0.7-kb DNA insert was recovered after EcoRI cleavage of the plasmid and agarose gel electrophoresis.

(33-2) Preparation of library and screening

The chromosomal DNA of *Ogataea minuta* IFO 10746 strain was cleaved with different restriction enzymes, and subjected to Southern analysis using the DNA fragment obtained in Example (33-1) as a probe by the method described in Example (2-2). The results suggested that there existed the *LEU2* gene in the BamHI-ClaI fragment of approximately 6 kb. Then, to clone the DNA fragment, a library was prepared. The chromosomal DNA of *Ogataea minuta* was cleaved with BamHI and ClaI and subjected to agarose gel electrophoresis, and then the approximately 6-kb DNA fragment was recovered from the gel. The recovered DNA fragment was ligated with BamHI- and ClaI-cleaved pBluescript II KS+ and then transformed into *Escherichia coli* strain DH5 α to prepare a library.

About 3,000 clones were screened by colony hybridization using the above described DNA fragment as a probe. A clone bearing plasmid pOMYP1 was selected from the 7 positive clones obtained.

(33-3) Sequencing of nucleotide sequence

The nucleotide sequence of the BamHI-ClaI region of the plasmid pOMLE1 (Fig. 28) was determined by primer walking method to obtain the nucleotide sequence represented by SEQ ID NO:107.

In the nucleotide sequence of SEQ ID NO:107, there existed an open reading frame of 1,089 bp, starting at position 1,606 and ends at position 2,694. The homology studies between the amino acid sequence (SEQ ID NO:108) deduced from the open reading frame and the *LEU2* gene product from *Saccharomyces cerevisiae* or *Pichia angusta* showed that 80% or 85% of amino acids were respectively identical between them.

Example 34

Preparation of *Ogataea minuta LEU2* knockout mutant

The *LEU2* gene was disrupted by transformation using the *URA3* gene of *Ogataea minuta* as a marker.

(34-1) Preparation of *LEU2* gene disruption vector

As shown in Fig. 28, plasmid pDOMLE1 was prepared by replacing the approximately 540-bp region of the *LEU2* structural gene by the *URA3* gene. To obtain a uracil auxotrophic mutant again from *LEU2* gene knockout mutants, the *URA3* gene having repetitive structures before and after the structural gene was used as a marker. The pROMUHT described in Example (31-1) was cleaved with HindIII, blunt-ended, and ligated with a NheI linker. The obtained plasmid was named pROMUNT.

The pOMLE1 was cleaved with StuI, blunt-ended, and ligated with a NheI linker. The obtained plasmid was named pOMLE2. The 3.3-kb Nhe-EcoT22I fragment isolated from the pOMURNT was inserted into the NheI-PstI of the pOMLE2. The obtained plasmid was named pDOMLE1.

(34-2) Transformation

The pDOMLE1 obtained in Example (34-1) was cleaved with BamHI and ClaI, and transformed into the *Ogataea minuta* TK11 strain (och1Δ ktrlΔ pep4Δ prb1Δ ura3Δ adelΔ) obtained in Example (17-2) by electric pulse method. To confirm that the *LEU2* gene of

these strains was disrupted, the following primers were synthesized (see Fig. 29 with regard to the position of each primer):

DL5; 5'-CAGGAGCTACAGAGTCATCG-3' (SEQ ID NO:109)

DL3; 5'-ACGAGGGACAGGTTGCTCGC-3' (SEQ ID NO:110)

PCR by primers DL5 and DL3 was performed using the chromosomal DNA isolated from the transformant as a template ((94°C for 30 seconds, 60°C for 1 minute and 72°C for 2 minutes) × 25 cycles). As shown in Fig. 29, a 4 kb amplified fragment was detected in the strain whose *LEU2* locus had the plasmid integrated thereinto. The selected strain was cultured on the YPD medium until stationary phase, and a strain resistant to 5-fluoroorotic acid (5-FOA) was obtained. PCR by primers DL5 and DL3 was performed using the chromosomal DNA of the 5-FOA resistant strain as a template ((94°C for 30 seconds, 60°C for 1 minute and 72°C for 3 minutes) × 25 cycles). As shown in Fig. 29, in the strain from which the *URA3* gene was deleted, a 1.6 kb amplified DNA fragment was detected. This och1Δ ktrlΔ pep4Δ prb1Δ ura3Δ adelΔ leu2Δ strain was named *Ogataea minuta* YK2.

Example 35

Construction of heterologous gene expression plasmid using *AOX1* gene promoter and terminator, and *LEU2* gene as a selectable marker

The pOMLE1 comprising the *LEU2* gene described in Example (33-2) was cleaved with PmaCI, ligated with an ApaI linker, cleaved with BamHI, blunt-ended, and ligated with a KpnI linker. The *LEU2* gene expression cassette was isolated, as a 3.3-kb ApaI-KpnI fragment, from the obtained plasmid, and then inserted into the ApaI-KpnI of the POMex1U. The obtained plasmid was cleaved with SpeI, blunt-ended, and ligated with a NotI linker. The resultant plasmid was named pOMex7L (Fig. 30).

The approximately 1.4-kb SpeI-BglII fragment comprising the *Saccharomyces cerevisiae*-derived invertase gene, obtained in Example 25, was inserted into the XbaI-BamHI of the pOMex7L to prepare pOMIV7L. This plasmid was cleaved with NotI and transferred into the *Ogataea minuta* YK2 strain described in Example (34-2). The transformant was cultured in the BYPM medium (0.67% yeast nitrogen base, 1% yeast extract, 2% polypeptone,

100 mM potassium phosphate buffer pH 6.0, 0.5% methanol). The culture was centrifuged and the supernatant was measured for invertase activity by the following procedures. Specifically, 2 μl of the culture supernatant appropriately diluted and 200 μl of 100 mM sodium acetate buffer (pH 5.0) containing 2% sucrose were mixed together and incubated at 37°C for 10-30 minutes, and 500 μl of Glucose-Test Wako (Wako Pure Chemical Industries, Inc., Japan) was added to the 2 μl of the reaction mixture to develop color. The absorbance based on free glucose generated by invertase was measured at 505 nm. In the most productive yeast strain *Ogataea minuta* YK2-IVL1, a significant amount of invertase was produced in the medium.

Example 36

Cloning of *YPS1* gene from *Ogataea minuta*

The *YPS1* gene was obtained from *Ogataea minuta* IFO 10746, and its nucleotide sequence was determined.

(36-1) Preparation of probe

Oligonucleotides having nucleotide sequences corresponding to the following amino acid sequences conserved in *YPS1* gene products from *Saccharomyces cerevisiae* (Accession number; NP_013221) and *Candida albicans* (Accession number; AAF66711):

DTGSSDLW (SEQ ID NO:111); and

FGAIDHAK (SEQ ID NO:112)

were synthesized as follows.

PLE5; 5'-GAYACNGGHTCNTCNGAYYTNTGG-3' (SEQ ID NO:113)

PLE3; 5'-TTYGGHGCNATYGAYCAYGCNAA-3' (SEQ ID NO:114)

The primer PYP5 has a sequence complementary to the nucleotide sequence corresponding to the amino acid sequence DTGSSDLW, and the primer PYP3 has a sequence complementary to the nucleotide sequence corresponding to the amino acid sequence FGAIDHAK.

PCR by primers PYP5 and PYP3 was performed using the chromosomal DNA of *Ogataea minuta* IFO 10746 as a template ((94°C for 30 seconds, 50°C for 1 minute and 72°C

for 1 minute) × 25 cycles). Approximately 0.6 kb amplified DNA fragment was recovered and cloned using TOPO TA Cloning Kit. Plasmid DNA was isolated from the obtained clone and sequenced. For a DNA insert of the plasmid, a clone was selected which had a nucleotide sequence encoding an amino acid sequence highly homologous to the amino acid sequences of *YPS1* gene products from *Saccharomyces cerevisiae* and *Candida albicans.* The 0.6-kb DNA insert was recovered after EcoRI digestion of the plasmid and agarose gel electrophoresis.

(36-2) Preparation of library and screening

The chromosomal DNA of *Ogataea minuta* IFO 10746 was cleaved with different restriction enzymes, and subjected to Southern analysis using the DNA fragment obtained in Example (36-1) as a probe by the method described in Example (2-2). The results suggested that there existed *YPS1* gene in the EcoRI fragment of approximately 4 kb. Then, to clone the DNA fragment, a library was constructed. The chromosomal DNA of the *Ogataea minuta* was cleaved with EcoRI and subjected to agarose gel electrophoresis, and then the approximately 6-kb DNA fragment was recovered from the gel. The recovered DNA fragment was ligated with EcoRI-cleaved and BAP-treated pUC118 and then transformed into *Escherichia coli* strain DH5 α to prepare a library.

About 2,000 clones were screened by colony hybridization using the above described DNA fragment as a probe. A clone bearing plasmid pOMYP1 was selected from the 4 positive clones obtained.

(36-3) Sequencing of nucleotide sequence

The nucleotide sequence of the EcoRI region of the plasmid pOMLE1 (Fig. 31) was determined by primer walking method to obtain a nucleotide sequence represented by SEQ ID NO:115.

In the nucleotide sequence of SEQ ID NO:115, there existed an open reading frame of 1,812 bp, starting at 1position 1,712 and ends at position 3,523. The homology studies between the amino acid sequence (SEQ ID NO:16) deduced from the open reading frame and

the *YPS1* gene product from *Saccharomyces cerevisiae* or *Candida albicans* showed that 40% or 27% of amino acids were respectively identical between them.

Example 37

Preparation of *Ogataea minuta YPS1* knockout mutant

The *YPS1* gene was disrupted by transformation using the *URA3* gene of *Ogataea minuta* as a marker.

(37-1) Preparation of YPS1 gene disruption vector

As shown in Fig. 31, plasmid pDOMYP1 was prepared by replacing the approximately 300-bp region of the *YPS1* structural gene by the *URA3* gene. To obtain a uracil auxotrophic mutant again from *YPS1* knockout mutants, the *URA3* gene having repetitive structures before and after the structural gene was used as a marker. The pROMUHT described in Example (31-1) was cleaved with HindIII, blunt-ended, and ligated with an EcoT22I linker. The obtained plasmid was named pROMUTT.

The pOMYP1 was cleaved with EcoRI, and the obtained fragment was ligated with EcoRI-cleaved and BAP-treated pBluescript II KS+. The obtained plasmid was named pOMYP2. This plasmid was cleaved with BsiWI and blunt-ended, and an EcoT22I linker was inserted thereinto. The obtained plasmid was named pOMYP3. The 3.3-kb EcoT22I fragment isolated from the pOMURTT was inserted at the EcoT22I of the pOMYP3. The obtained plasmid was named pDOMYP1.

(37-2) Transformation

The pDOMYP1 obtained in Example (37-1) was cleaved with BamHI and ClaI, and transformed into the *Ogataea minuta* TK11 strain (och1Δ ktrlΔ pep4Δ prb1Δ ura3Δ adelΔ) obtained in Example (17-2) by electric pulse method. To confirm that the *YPS1* gene was disrupted, the following primers were synthesized (see Fig. 32 with regard to the position of each primer).

DY5; 5'-CTCAAGGGCCTGGAGACTACG-3' (SEQ ID NO:117)

DY3; 5'-CGGGATTCCCGAGTCGCTCACC-3' (SEQ ID NO:118)

PCR by primers DY5 and DY3 was performed using the chromosomal DNA isolated from the transformant as a template ((94°C for 30 seconds, 60°C for 1 minute and 72°C for 2 minutes) × 25 cycles). As shown in Fig. *8*, a 3.7 kb amplified DNA fragment was detected in the strain whose *YPS1* locus had the plasmid integrated thereinto. The selected strain was cultured on the YPD medium until stationary phase, and a strain resistant to 5-fluoroorotic acid (5-FOA) was obtained. PCR by primers DY5 and DY3 was performed using the chromosomal DNA of the 5-FOA resistant strain as a template ((94°C for 30 seconds, 60°C for 1 minute and 72°C for 3 minutes) × 25 cycles). As shown in Fig. 32, a 1.2 kb amplified DNA fragment was detected in the strain from which the *URA3* gene was deleted. This och1Δ ktrlΔ pep4Δ prb1Δ ura3Δ adelΔ yps1Δ strain was named *Ogataea minuta* YK3.

Example 38

Transferring of human antibody gene into *Ogataea minuta YPS1* knockout mutant and expression of same

Human G-CSF light chain gene (SEQ ID NO:91) and heavy chain gene (SEQ ID NO:92) were transferred into the *Ogataea minuta* YK3 strain (och1Δ ktrlΔ pep4Δ prb1Δ ura3Δ adelΔ yps1Δ) obtained in Example (37-2). The plasmid vector expressing anti-G-CSF light chain and heavy chain genes, described in Example 28, was cleaved with NotI, the *Ogataea minuta* YK3 strain was transformed in turn. In accordance with the method described in Example 28, a transformant that produced the antibodies in the culture supernatant was selected from the obtained transformants, and the *Ogataea minuta* YK3-derived antibody producing strain was named *Ogataea minuta* YK3-IgB1.

Then *Aspergillus saitoi*-derived α-1,2-mannosidase gene was transferred into the *Ogataea minuta* YK3-IgB1 strain. After transformation using the plasmid pOMaM1U prepared in Example 23 by the method described in Example 24, an α-1,2-mannosidase expressing strain was selected from the obtained transformants. The resultant strain was named *Ogataea minuta* YK3-IgB-aM. The *Ogataea minuta* YK3-IgB-aM strain and the *Ogataea minuta* TK9-IgB-aM strain prepared in Example 28 as a control were cultured in the BYPMG medium at 28°C for 72 hours and centrifuged. The culture supernatant obtained by

the centrifugation was subjected to Western analysis. The results are shown in Fig. 33. The results revealed that in antibody molecules produced by the *Ogataea minuta* TK9-IgB-aM strain, as a control, molecules with degraded heavy chains were detected, whereas in the antibody molecules produced by the *Ogataea minuta* YK3-IgB-aM strain, the degradation of the heavy chains was retarded.

Further, the culture supernatant of the *Ogataea minuta* YK3-IgB-aM strain was concentrated by ultrafiltration using an Amicon YM76 membrane (Amicon), desalted, and subjected to Protein A column chromatography (Hi-Trap ProteinA HP, Amersham Pharmacia Biotech) to purify the antibody fractions through the elution with glycine – HCl, pH 3.0. Western analysis was performed for the purified antibody samples (Fig. 34). The results of SDS-PAGE under non-reducing conditions, it was found that a full-length antibody molecule, which was composed mainly of two light chain molecules and two heavy chain molecules, was produced. The binding of the purified antibody to G-CSF was confirmed by the method described in Example 28. The antibody was dialyzed and freeze-dried. PA-N-linked sugar chains were prepared by the method described in Example 11 and subjected to size analysis by normal phase column. From the results, it was confirmed that the sugar chain of the antibody contained $Man_5GlcNAc_2$, which was a mammalian and high mannose type sugar chain.

Example 39

Transferring of a molecular chaperone Protein Disulfide Isomerase (PDI) gene into human antibody producing strain prepared in Example 38, and expression of same

The results obtained above confirmed that the *Ogataea minuta* YK3-IgB1-aM strain produced only a trace amount of the antibody in the culture supernatant, while the results of the Western analysis revealed that a significant amount of the antibody was accumulated in the cells (Fig. 35, lanes 1, 5). As it was presumed that the antibody protein was not fully folded, we attempted to express Protein Disulfide Isomerase (*PDI*) gene, as a molecular chaperone. To express the *PDI* gene, we constructed a plasmid, which expressed *PDI* gene using *AOX1* gene promoter and a hygromycin resistant gene as a selectable marker.

To obtain the *PDI* gene (M62815) from *Saccharomyces cerevisiae*, the following primers corresponding to the N-and C-termini of the *PDI* were synthesized.

PDI5; 5'-TCTAGAATGAAGTTTTCTGCTGGTGCCGTCCTG-3' (SEQ ID NO:119)

PDI3; 5'-GGATCCTTACAATTCATCGTGAATGGCATCTTC-3' (SEQ ID NO:120)

PCR by primers PDI5 and PDI3 was performed using the chromosomal DNA of *Saccharomyces cerevisiae* S288C as a template ((94°C for 30 seconds, 55°C for 1 minute and 72°C for 1 minute) × 20 cycles). 1.5 kb amplified DNA fragment was recovered and cloned using TOPO TA Cloning Kit. The nucleotide sequence of the DNA insert was determined and a clone having the correct nucleotide sequence was selected. The *PDI* gene of *Saccharomyces cerevisiae* can be isolated as a SpeI-BamHI fragment.

Then, the XbaI-BamHI fragment comprising the above-described *PDI* gene was inserted into the XbaI-BamHI of the expression cassette using the *Ogataea minuta AOX1* gene promoter and terminator, as prepared in Example (21-5), and the expression plasmid pOMex5H comprising the hygromycin resistant gene as a selectable marker. The resultant plasmid was named pOMex5H-PDI.

The pOMex5H-PDI was cleaved with NotI, and the *Ogataea minuta* YK3-IgB1-aM strain was transformed therewith. The transformants were cultured in the BYPMG medium and centrifuged, the culture supernatant obtained by the centrifugation was subjected to Western analysis in the same manner as in Example 38, and a transformant that produced the antibody in the culture supernatant was selected. The *Ogataea minuta* YK3-IgB-aM-derived antibody producing strain was named *Ogataea minuta* YK3-IgB-aM-P. The *Ogataea minuta* YK3-IgB-aM-P strain produced a significant amount of the full-length antibody molecule as compared with the original strain *Ogataea minuta* YK3-IgB-aM into which no molecular chaperon was transferred (Fig. 35, lane 4), and in which the amount of antibody accumulated in the cells was decreased (Fig. 35, lane 6).

The antibody fractions were purified from the culture supernatant of the *Ogataea minuta* YK3-IgB-aM strain by the method described in Example 38. The antibody fractions were dialyzed and freeze-dried. PA-N-linked sugar chains were prepared by the method described in Example 11, and subjected to size analysis by normal phase column to confirm

that the sugar chain of the antibody produced by the *Ogataea minuta* YK3-IgB-aM strain contained $Man_5GlcNAc_2$, which was a mammalian type, high mannose type sugar chain.

Industrial Applicability

Using the methylotrophic yeast carrying a sugar chain mutation, which is newly prepared by genetic engineering techniques of the invention, a neutral sugar chain identical with a high mannose type sugar chain produced by mammalian cells such as human cells, or a glycoprotein having the same neutral sugar chain, can be produced in a large amount at a high purity. Further, by transferring a mammalian type sugar chain biosynthesis-associated gene(s) into the above described mutant strain, a hybrid type or complex type mammalian sugar chain or a protein comprising mammalian type sugar chain can be efficiently produced. The yeast strains and glycoproteins of the invention are applicable to medicaments, etc..

The disclosure of all the publications, patents and patent applications cited herein is incorporated herein by reference.